UBR E3 ligases and the PDIA3 protease control degradation of unfolded antibody heavy chain by ERAD

Danming Tang, Wendy Sandoval, Cynthia Lam, Benjamin Hayley, Peter Liu, Di Xue, Deepankar Roy, Tom Patapoff, Salina Louie, Brad Snedecor, and Shahram Misaghi

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September 10, 2019

Re: JCB manuscript #201908087

Dr. Shahram Misaghi
Genentech Inc.
1 DNA Way
South San Francisco, CALIFORNIA 94080

Dear Dr. Misaghi,

Thank you for submitting your manuscript entitled "UBR4 and UBR5 together with Protease PDIA3 Mediate the ERAD of Unfolded Antibody Heavy Chains". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers found the molecular characterization of a new ERAD pathway for HC proteins interesting but preliminary. This is the aspect of greatest potential interest to cell biologists and we agree with the referees' assessments that the mechanistic studies and conclusions need to be deepened and strengthened for publication in JCB. We find the reviewers' criticisms and questions valid and feel that they need to be addressed in full. The referees provide constructive comments that will provide a more definitive understanding of the role of the catalytic activity of UBR4/5 and PDIA3, the precise membrane localization of the suggested pathway, the contribution of the cleavage and ubiquitination steps, and of the roles of Hrd1 or other E3s.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript by Tang et al., the authors identified two cytosolic membrane-associated E3 ubiquitin ligases, UBR4 and UBR5, that ubiquitinate the antibody heavy chain (HC) during ERAD retrotranslocation, thereby targeting HC for proteasomal degradation. Additionally, the investigators also found the ER luminal protease PDIA3 cleaves ubiquitinated HC, a reaction essential for release of the substrate to the proteasome for degradation. The identification of the PDIA3-UBR4/5 axis during ERAD retrotranslocation is novel and interesting, and would add further insight into this critical protein quality control pathway. However, additional experiments are required to robustly support the model (presented in Figure 5). Specifically:

Major points:

1. A cycloheximide (CHX) chase (or radioactive pulse-chase) experiment is the "gold standard" approach to evaluate protein turnover of ERAD substrates. This should be done under both UBR4/5 and PDIA3 conditions (for both Figures 2 and 4).

2. The model depicted in Figure 5 is that the 55 kDa HC is cleaved by PDIA3, generating an N-terminal 35 kDa N-terminal HC fragment, and presumably a 20 kDa C-terminal HC fragment. Data provided in this paper suggests that the 35 kDa N-terminal HC fragment is retrotranslocated and ubiquitinated by UBR4/5. What is the fate of the 20 kDa C-terminal HC fragment? Does UBR4/5

also ubiquitinate this species? Simply tagging the C-terminus of the HC would allow one to follow its fate, especially using the aforementioned CHX assay. This is a relevant point because UBR4/5 have been implicated in N-degron recognition, raising the question of whether the C-terminal HC fragment might be a substrate of these E3 ligases (as suggested in the model of Figure 5).

3. The idea that the HC is ubiquitinated by UBR4/5 is intriguing, given that the canonical model of ERAD suggests that the E3 ligase activity of the retrotranslocon itself such as Hrd1 is responsible for substrate ubiquitination. In light of this,

A. Does ERAD of the HC dependent on Hrd1? gp78? MARCH6?
B. If Hrd1, is the E3 ligase activity of Hrd1 important for ERAD of the HC?
C. If not, this would be equally interesting because it suggests that the ligase activity of UBR4/5 might provide the source of substrate ubiquitination.

I think the authors should better flush out this area of investigation to give stronger mechanistic insight into ERAD retrotranslocation of the HC.

Minor points:

1. Figures 6 and 7 are not central to this manuscript, and should be omitted.

2. In Figure 4F, a FLAG blot should be provided to reveal the level of precipitated HC.

3. Does PDIA3 cleave other ERAD substrates?

Reviewer #2 (Comments to the Authors (Required)):

UBR4 and UBR5 are known as N-recognins of the N-degron pathway that recognize and bind single N-terminal residues of proteins to mediate ubiquitination and proteasomal degradation. In the current study, the authors propose that UBR4 and UBR5 play a key role in ubiquitination and proteasomal degradation of unfolded antibody heavy chain (HC) and that the cysteine protease PDIA3 cleaves ubiquitinated-HC molecules to accelerate HC dislocation and ubiquitination as well as proteasomal degradation. Overall, the major claims are supported by a set of carefully designed experiments. The results from this study provide critical information in the fields of the N-degron pathway and ER protein quality control, and possibly in the industry section as well. Nonetheless, there are also some concerns and comments that should be addressed before considering for publication.

Main points

1. It is unclear whether UBR4 and UBR5 directly recognize misfolded HC peptides or indirectly through the assistance of ER-residing chaperones. How do UBR4 and UBR5 selectively recognize HCs but not general ER clients of ERAD? These comments are optional, but I like to see how the authors address them.
2. Another question would be whether UBR4 and UBR5 recognize N-degrons exposed on misfolded HCs. Would it be possible to produce misfolded HC substrates carrying N-terminal arginine in comparison with a stabilizing residue such as valine? This comment is also optional.
3. For Fig 2E and 2F, compared to the total amount of poly-Ub chains conjugated to the antibody

heavy chain, the amount of K48-linked poly-Ub chains do not likely seem to represent total ubiquitin. Likewise, UBR4/5 knockdown reduces not only K48-linked poly-Ub chains, but also K63- and K11-linked chains ligated on HC. The authors should investigate more, hopefully all seven, lysine Ub mutants to find the unaffected chain linkage type to validate their claims.
4. For Fig 4C, the levels of mAb2 HC under triple knockdown of UBR4, UBR5 and PDIA3 should be the largest if UBR4/5 and PDIA3 works cooperatively as the authors suggest. However, the levels of mAb2 HC under triple knockdown seem lower than that under PDIA3 single knockdown. The authors should clarify this point.
5. For Fig 4F, ubiquitination levels on HC/LC were decreased upon PDIA3 knockdown even in UBR4/5 deficient conditions. The authors should clarify whether PDIA3 by itself or in tandem with other ligases is sufficient to degrade the misfolded HC.

Minor points

1. In Fig 3A, Tyrosine 212 should be changed to Threonine 212.
2. Although the authors claimed that HC translocon and proteasomal degradation are coupled, Fig 1B showed only a marginal proteasomal degradation flux of HC in membrane fraction.
3. For Fig 1B, it seems a little weird that the originally cytosolic protein UBR4 and UBR5 are seemingly completely relocated to the membrane fraction without a trace left in the cytosolic fraction.
4. It is recommended that "the N-end rule pathway" is replaced with the newly coined official terminology, namely "the N-degron pathway."
5. The authors should cite the original primary paper (Tasaki et al., 2005) when introducing UBR4 and UBR5 as N-recognin E3 ligases
6. The authors should mention the concentration of siRNA oligo used in Materials and Methods section or in Figure legends.

Reviewer #3 (Comments to the Authors (Required)):

Antibodies represent a major biopharmaceutical agent, and their manufacturing is a huge commercial enterprise. Thus, understanding how to optimize their production is an important goal. Antibodies have also been the focus of numerous basic science studies conducted using in vivo and in vitro systems. Therefore, a study on ERQC of antibodies is of broad interest to basic and applied research. The authors present data to suggest that two N-end rule Ub ligases, UBR4 and UBR5, are critical for proteasomal degradation of Ig γ heavy chains and that PDIA3 serves to enhance degradation by cleaving the γ heavy chains near the end of the CH1 domain, which is the focus of ERQC for antibodies. While the data are potentially interesting, a number of the findings in this manuscript are inconsistent with published data, critical controls are often missing, the literature on this subject are poorly cited, and the conclusions in some cases are difficult to understand or hard to justify. The last two figures using normal B cell populations do not add much to the manuscript and could be deleted. In addition, the authors should more clearly describe seed train and production cultures.

General points:
1. The E3 ligase UBR4 is reported to bind microtubules, and UBR5 has been localized to the nucleus, although neither localization was referenced. Is it possible that the observation of UBR4/5 in their "membrane" fraction is due to contamination of nuclear proteins and microtubules in their hypotonic disruption protocol? Experiments to show direct localization of these proteins to the ER

membrane are needed, as I was unable to find anything in the literature to support ER location of either protein.

2. A truncated Ig γ heavy chain has been shown to be modified by pH-sensitive Ub linkages, and the blots presented in this manuscript show a very broad pattern for Ub of the γ heavy chain, which is consistent with more than a single N-terminal Ub chain attachment. Again, no reference on this point. It is possible that the N-terminal attachment is required to pull the chain far enough through the retrotranslocon to allow sequential ubiquitin chain attachments to other residues. It would further the observations reported here if the authors mutated the client to an N-terminal amino acid that cannot be arginylated and demonstrated that it was no longer ubiquitinated and was stabilized. Similarly, checking to see if a significant amount of the ubiquitin signal is pH-sensitive could provide a more complete understanding of ERAD for this client and would do nothing to distract from this manuscript.

3. The possibility that a protease cleaves the N-terminal half of the HC protein to simplify the clearing of ophan subunits is intriguing. However, based on multiple published studies, one would expect that the C-terminal "hinge-CH2-CH3" fragment, which should be folded and assembled into dimers, would then be secreted, while the VH-CH1 fragment would be targeted for degradation due to the unfolded CH1 domain. However, the data presented here do not include both the full-length protein and the C-terminal fragment on their blots of the media. Do the authors have an explanation as to why ERQC for the full-length γ heavy chain is so poor in this system?

4. PDIA3, aka ERp57, is the oxido-reductase associated with calnexin. Calnexin is not the primary chaperone for unassembled γ heavy chains, since the only stably unfolded domain in this protein should be the CH1 domain, which is not glycosylated. So even if PDIA3 has protease activity (the published literature on this possibility is very limited), it is surprising that it would be targeted to γ heavy chain. I recognize that the mutational analyses were meant to distinguish between isomerization and protease activity, but the same mutations used to inhibit proteinase activity should also inhibit reductase activity. Thus, it would be worthwhile to see if PDIA3 is acting to reduce the HC dimers in this system to aid in their extraction.

Other points:

Figure 2. What is the nature of the high molecular weight heavy chain species? It is about twice the size of the γ heavy chain Is this possibly a non-specific protein recognized by the anti-Flag antibody? The authors mention that the lines producing only HC are unstable and rapidly lose HC expression. Some of these chain-loss lines would make great controls for their experiments. All of the bands detected by anti-Flag should be part of a single image instead of shown as individual panels.

Figure 3. The single cleavage site very near the end of the CH1 domain is intriguing. Again, the full-length HC and HC fragment should be on a single blot instead of being shown as separate panels. Importantly, the CH1 domain is unstructured when unassembled. The authors also show similar amounts of this cleavage product in cultures from cells expressing both HC and LC. Are these generated only from unassembled HC, or is the site in the CH1 domain cleaved even after assembly with LC? Based on the structure presented, it seems the authors might be implying this. However, the perceived lack of specificity between unassembled and assembled heavy chains for this protease are difficult to model into a role in ERQC.

Figures 4E and F provide the only images in which the full-length HC and HC fragment are shown in

a single panel. In 4E, both forms are present in near equal levels under control, steady-state conditions. This is surprising if the cleaved fragment is an intermediate in ERAD. Pulse-chase experiments would go a long way to prove precursor product relationship. However, in 4F there is very little of the fragment and nearly all of the HC is present as the high molecular weight form. Is it possible this high molecular weight band is non-specific? The ubiquitin signals should be normalized to the amount of HC for each treatment. Was this done in the presence of a proteasome inhibitor? There is nothing in the text or legend to indicate this. In Figure 4C and 4E, the fragment disappears when PDIA3 is knockdown alone, but large amounts of it are generated when both the E3s and PDIA3 are knocked down together. How do the authors reconcile this data if PDIA3 is the protease?

Dear Editor,

Many thanks for your consideration to publish our manuscript “UBR4 and UBR5 together with Protease PDIA3 Mediate the ERAD of Unfolded Antibody Heavy Chains”. Here we have addressed all the reviewers’ comments point by point, hopefully to their and your satisfaction, and revised our manuscript accordingly. We are now submitting the revised version of the manuscript together with 6 figures and 1 supplementary file. You may find all the relevant files on the website.

Thank you again for your consideration.

Sincerely,
Danming Tang

September 10, 2019

Re: JCB manuscript #201908087

Dr. Shahram Misaghi
Genentech Inc.
1 DNA Way
South San Francisco, CALIFORNIA 94080

Dear Dr. Misaghi,

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Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data
Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

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Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript by Tang et al., the authors identified two cytosolic membrane-associated E3 ubiquitin ligases, UBR4 and UBR5, that ubiquitinate the antibody heavy chain (HC) during ERAD retrotranslocation, thereby targeting HC for proteasomal degradation. Additionally, the investigators also found the ER luminal protease PDIA3 cleaves ubiquitinated HC, a reaction essential for release of the substrate to the proteasome for degradation. The identification of the PDIA3-UBR4/5 axis during ERAD retrotranslocation is novel and interesting, and would add further insight into this critical protein quality control pathway. However, additional experiments are required to robustly support the model (presented in Figure 5). Specifically:

Major points:

1. A cycloheximide (CHX) chase (or radioactive pulse-chase) experiment is the "gold standard" approach to evaluate protein turnover of ERAD substrates. This should be done under both UBR4/5 and PDIA3 conditions (for both Figures 2 and 4).

We thank the reviewer for this suggestion. We have added a panel showing the CHX chase assay for both control cells and the UBR4/5 knock-down cells as Supplementary Figure 3C. The result showed that when UBR4/5 were knocked down in the cell, the clearance of IgG HC was slowed down and a concurrent accumulation of HC fragment was observed, suggesting that degradation of the cleaved HC molecules is one of the major clearance routes for HC removal from the ER in these cells. The anti-human Fc antibody seems to have a stronger affinity to HC fragment than HC, therefore the band intensity of the HC fragment is stronger than the decrease in the band intensity fo the full-length HC. Also, keep in mind that the overall degradation rate of the HC molecule was relatively slow, as in a 24-hour CHX treatment (data not shown).

We have also added a panel (Supplementary Figure 5B) to track the HC cleavage and role of PDIA3 in this process. The result shows that in CHX chase, further addition of PDIA3 inhibitor pHMB prevented accumulation of HC fragment and to a great degree reduced degradation rate of the full-length HC. Note that in this experiment the degradation rate of a subpopulation of the full-length HC molecules that may get degraded without undergoing cleavage remains unaffected. This likely accounts for the observed slow degradation of HC species even in the presence of pHMB. All together, we believe that the results clearly show that both UBR4/5 mediated ubiquitination and degradation and PDIA3 mediated cleavage are involved in mis-folded HC clearance. These two mechanisms may function independently and/or can work together to orchestrate degradation of misfolded HC molecules.

2. The model depicted in Figure 5 is that the 55 kDa HC is cleaved by PDIA3, generating an N-terminal 35 kDa N-terminal HC fragment, and presumably a 20 kDa C-terminal HC fragment. Data provided in this paper suggests that the 35 kDa N-terminal HC fragment is retrotranslocated and ubiquitinated by UBR4/5. What is the fate of the 20 kDa C-terminal HC fragment? Does UBR4/5 also ubiquitinate this species? Simply tagging the C-terminus of the HC would allow one to follow its fate, especially using the aforementioned CHX assay. This is a relevant point because UBR4/5 have been implicated in N-degron recognition, raising the question of whether the C-terminal HC fragment might be a substrate of these E3 ligases (as suggested in the model of Figure 5).

Our HC molecules are tagged at the C-terminus (see supplementary Figure-1A). After the full-length HC is recognized and ubiquitinated by UBR4/5, PDIA3 cleaves the ubiquitinated HC in the middle and generates a 20 kDa N-terminal fragment and a 35kDa C-terminal fragment. The 20 kDa fragment is the N-terminal fragment, it is ubiquitinated and removed from the ER by proteasome immediately after the cleavage. The 35kDa C-terminal fragment is free in the ER and will also be degraded by UBR4/5 later. We occasionally can observe the N-terminal 20kDa fragment when blotting with anti-human Fc antibody, but not always, perhaps due to the faster degradation rate of the N-terminal fragment.

3. The idea that the HC is ubiquitinated by UBR4/5 is intriguing, given that the canonical model of ERAD suggests that the E3 ligase activity of the retrotranslocon itself such as Hrd1 is responsible for substrate ubiquitination. In light of this,

A. Does ERAD of the HC dependent on Hrd1? gp78? MARCH6?
B. If Hrd1, is the E3 ligase activity of Hrd1 important for ERAD of the HC?
C. If not, this would be equally interesting because it suggests that the ligase activity of UBR4/5 might provide the source of substrate ubiquitination.

We have tried to deplete Hrd1, gp78, MARCH6 or TRC8. Unfortunately, we did not have good antibodies against MARCH6 and TRC8 to determine the knock-down efficiency (keep in mind that most of the antibodies are developed to recognize mice and human proteins), although transfection of these siRNAs did not increase HC level in the cells. Knock-down of gp78 to 50% did not change HC level, while knock-down of Hrd1 increased HC level to a similar extent of UBR4/5 knockdown. Knock-down of UBR4, UBR5 and Hrd1 altogether further increased HC level, suggesting the possibility that Hrd1 and UBR4/UBR5 can ubiquitinate and mark HC degradation separately. This result is now shown as Figure 2E and supplementary figure 3D. These findings confirm the role of UBR4/5 in degradation of HC molecules from ER. While we cannot yet fully rule out whether UBR4/5 and Hrd1 function independently or in concert, further increase in HC accumulation when all 3 proteins are knocked down simultaneously hints that they might likely function independently.

I think the authors should better flush out this area of investigation to give stronger mechanistic insight into ERAD retrotranslocation of the HC.

In Page 22 of the result section and page 32 of the discussion section we added a couple of paragraphs expanding upon roles of UBR4/5 and Hrd1 and other E3 ligases in HC ERAD.

Minor points:

1. Figures 6 and 7 are not central to this manuscript, and should be omitted.

We thank the reviewer for this suggestion. We have omitted the majority of these two figures and combined the rest into one half-page figure since these data are of interest to researchers in the pharmaceutical industry. Since improving antibody productivity in CHO cells is of great interest for protein manufacturing, we would like to keep these results for the readers in this area.

2. In Figure 4F, a FLAG blot should be provided to reveal the level of precipitated HC.
Fig 4E showed the level of precipitated HC. We have modified the figure legends to clarify that Figure 4C-F were from one experiment.

3. Does PDIA3 cleave other ERAD substrates?
Qiu et al., 2004 has shown that ApoB100 is cleaved by PDIA3, which generate a 50kDa fragment. We have added this in the discussion in page 33.

Reviewer #2 (Comments to the Authors (Required)):

UBR4 and UBR5 are known as N-recognins of the N-degron pathway that recognize and bind single N-terminal residues of proteins to mediate ubiquitination and proteasomal degradation. In the current study, the authors propose that UBR4 and UBR5 play a key role in ubiquitination and proteasomal degradation of unfolded antibody heavy chain (HC) and that the cysteine protease PDIA3 cleaves ubiquitinated-HC molecules to accelerate HC dislocation and ubiquitination as well as proteasomal degradation. Overall, the major claims are supported by a set of carefully designed experiments. The results from this study provide critical information in the fields of the N-degron pathway and ER protein quality control, and possibly in the industry section as well. Nonetheless, there are also some concerns and comments that should be addressed before considering for publication.

Main points

1. It is unclear whether UBR4 and UBR5 directly recognize misfolded HC peptides or indirectly through the assistance of ER-residing chaperones. How do UBR4 and UBR5

selectively recognize HCs but not general ER clients of ERAD? These comments are optional, but I like to see how the authors address them.

We currently have no evidence to suggest how UBR4 and UBR5 recognize misfolded HC, it may be through chaperones and adaptors. Besides HC, other ER clients may be recognized by N-recognins. There is a publication from Shim et al. 2018 suggesting that in ER stress BiP is N-terminal arginylated degraded in N-degron pathway. We have tried to test this possibility in the CHO cells, however the antibody that recognizes arginylated-BiP in that paper did not work in CHO cells.

2. Another question would be whether UBR4 and UBR5 recognize N-degrons exposed on misfolded HCs. Would it be possible to produce misfolded HC substrates carrying N-terminal arginine in comparison with a stabilizing residue such as valine? This comment is also optional.

While we agree that it would be interesting to test the degradation speed of HC with different first amino acid. We do not believe that it would not be relevant to understanding the degradation of HC in our CHO system since using clinically tested and confirmed signal peptides and relevant first amino acid plays a critical role in expression of properly processed and expressed antibodies. As our CHO system is developed to express antibodies at very high titers and productivity rates, changing the framework signal peptide aa sequence would introduce many wrinkles for titer and product quality comparability criteria, which renders degradation rate comparisons invalid. For example, a change in first aa sequence can result in improper signal peptide cleavage or other product quality changes that in turn can change the HC flux within the ER, Golgi, and ultimately its secretion and degradation rates. Without extensively comparing and confirming effects of aa change in the vicinity of signal peptide (which is not in the scope of this study), it would be very difficult to draw clear conclusions from such studies.
That said, we do believe that all 20 amino acids can be subjected to N-degron pathway and valine is known to be another Ac/N-degron just as threonine.

3. For Fig 2E and 2F, compared to the total amount of poly-Ub chains conjugated to the antibody heavy chain, the amount of K48-linked poly-Ub chains do not likely seem to represent total ubiquitin. Likewise, UBR4/5 knockdown reduces not only K48-linked poly-Ub chains, but also K63- and K11-linked chains ligated on HC. The authors should investigate more, hopefully all seven, lysine Ub mutants to find the unaffected chain linkage type to validate their claims.

We thank the reviewer for this comment and have used mass-spectrometry to analyze different ubiquitinated chains that could be detected on the HC molecules. The mass spec data showed that the major ubiquitin linkage is K48 linked, and UBR4/5 depletion seems to reduce the level of K48 ubiquitination linkage of HC. There were some other minor poly-ubiquitin linkages detected on HC as well. The result is now shown as supplementary Figure 3E-F.

4. For Fig 4C, the levels of mAb2 HC under triple knockdown of UBR4, UBR5 and PDIA3 should be the largest if UBR4/5 and PDIA3 works cooperatively as the authors suggest. However, the levels of mAb2 HC under triple knockdown seem lower than that under PDIA3 single knockdown. The authors should clarify this point.

This is a very good question. We believe the higher levels of HC accumulation in PDIA3 single KD is due to these HC molecules are being stuck at the translocon and can not be cleared by the ERAD. In UBR4/BR5/PDIA3 triple KD cells, the full length HC molecules are not ubiquitinated at the N-terminus and can therefore move away from the translocon back into the ER lumen and be cleared by through secretion. We have added the explanation in page 27 of the results section.

5. For Fig 4F, ubiquitination levels on HC/LC were decreased upon PDIA3 knockdown even in UBR4/5 deficient conditions. The authors should clarify whether PDIA3 by itself or in tandem with other ligases is sufficient to degrade the misfolded HC.

In this experiment, the decrease of ubiquitination level was because of UBR4/5 KD. However, KD of PDIA3 increased HC ubiquitination in both UBR4/5 intact and deficient conditions, because the intact ubiquitinated HC molecules could be trapped in the translocon and could not be efficiently cleared. PDIA3 cleavage helps to accelerate the extraction of the N-terminal ubiquitinated HC fragments. KD of PDIA3 stabilizes ubiquitinated HC therefore causing an increase of ubiquitinated HC level. We have also edited the text to make this point clearer in page 26 of the results section.

Minor points

1. In Fig 3A, Tyrosine 212 should be changed to Threonine 212.

Thanks for the correction. We have changed the text accordingly.

2. Although the authors claimed that HC translocon and proteasomal degradation are coupled, Fig 1B showed only a marginal proteasomal degradation flux of HC in membrane fraction.

We agree with the reviewer that there is only a marginal proteasomal degradation flux of HC in 12 hours. However, we believe this is due to the turnover rate of unfolded HC, which is fairly slow, as observed in the CHX treatment experiment where even after 24 hours of CHX treatment the ratio of remaining HC to total intracellular proteins was comparable or even higher, suggesting that the degradation rate of HC is slower than most intracellular proteins (data not shown).

3. For Fig 1B, it seems a little weird that the originally cytosolic protein UBR4 and UBR5

are seemingly completely relocated to the membrane fraction without a trace left in the cytosolic fraction.

We have performed a detailed subcellular fractionation experiment, in supplementary figure 2, which showed that UBR4 and UBR5 can be found in both cytosol and ER in CHO cells. Also, the shear stress during the homogenization and the ionic strength of the homogenization buffer could affect the subcellular fractionation result shown here. For example, with increased mechanical shearing, as shown in supplementary figure 2, we observed a fraction of UBR4/5 were cytosolic (as the reviewer predicted to be for UBR4/5, which are cytosolic), while the Figure 1B the mechanical shear stress was likely less stringent. Note that UBR4/5 are not membrane or ER proteins, and only associated with the exterior membranes.

4. It is recommended that "the N-end rule pathway" is replaced with the newly coined official terminology, namely "the N-degron pathway."

We thank the reviewer for the suggestion. We have changed the text accordingly.

5. The authors should cite the original primary paper (Tasaki et al., 2005) when introducing UBR4 and UBR5 as N-recognin E3 ligases

We thank the reviewer for the suggestion. We have added this reference.

6. The authors should mention the concentration of siRNA oligo used in Materials and Methods section or in Figure legends.

We have added this information as requested.

Reviewer #3 (Comments to the Authors (Required)):

Antibodies represent a major biopharmaceutical agent, and their manufacturing is a huge commercial enterprise. Thus, understanding how to optimize their production is an important goal. Antibodies have also been the focus of numerous basic science studies conducted using in vivo and in vitro systems. Therefore, a study on ERQC of antibodies is of broad interest to basic and applied research. The authors present data to suggest that two N-end rule Ub ligases, UBR4 and UBR5, are critical for proteasomal degradation of Ig γ heavy chains and that PDIA3 serves to enhance degradation by cleaving the γ heavy chains near the end of the CH1 domain, which is the focus of ERQC for antibodies. While the data are potentially interesting, a number of the findings in this manuscript are inconsistent with published data, critical controls are often missing, the literature on this subject are poorly cited, and the conclusions in some cases are difficult to understand or hard to justify.

The last two figures using normal B cell populations do not add much to the manuscript and could be deleted. In addition, the authors should more clearly describe seed train and production cultures.

The last two figures are mainly focused on CHO cells expressing recombinant IgG, rather than normal B cell populations. We thank the reviewer for this suggestion. We have omitted the majority of these two figures and combined the rest into one small figure. However, the original purpose of this study was to improve antibody productivity in CHO cells for industry and manufacturing purposes and we would like to keep these results for the readers in this area.

General points:
1. The E3 ligase UBR4 is reported to bind microtubules, and UBR5 has been localized to the nucleus, although neither localization was referenced. Is it possible that the observation of UBR4/5 in their "membrane" fraction is due to contamination of nuclear proteins and microtubules in their hypotonic disruption protocol? Experiments to show direct localization of these proteins to the ER membrane are needed, as I was unable to find anything in the literature to support ER location of either protein.

We have performed a more detailed subcellular localization to show that UBR4 and UBR5 do localize on the ER membrane as well as in the cytosol, but not much in the nuclear. The paper that showed UBR5 nuclear localization was expressing GFP-tagged UBR5 in the cells, and it is known that GFP tag sometimes direct the fusion protein to the nucleus.

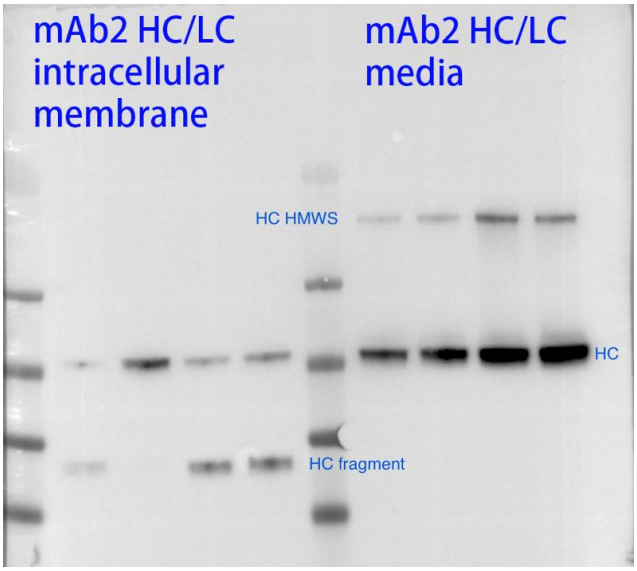
2. A truncated Ig γ heavy chain has been shown to be modified by pH-sensitive Ub linkages, and the blots presented in this manuscript show a very broad pattern for Ub of the γ heavy chain, which is consistent with more than a single N-terminal Ub chain attachment. Again, no reference on this point. It is possible that the N-terminal attachment is required to pull the chain far enough through the retrotranslocon to allow sequential ubiquitin chain attachments to other residues. It would further the observations reported here if the authors mutated the client to an N-terminal amino acid that cannot be arginylated and demonstrated that it was no longer ubiquitinated and was stabilized. Similarly, checking to see if a significant amount of the ubiquitin signal is pH-sensitive could provide a more complete understanding of ERAD for this client and would do nothing to distract from this manuscript.

The reviewer raised a very good point that the N-terminal ubiquitination is possibly the first step to pull HC out of translocon to allow further ubiquitination by UBR4/5 and other E3 ligases like Hrd1. We have now cited the paper the reviewer mentioned. Thank the reviewer for the suggestion. Based on published literatures, all 20 amino acids can be N-degrons. Mutating the first amino acid may change the degradation speed, but will not change the fact that UBR4/5 recognize HC as one of their ERAD substrates and HC most likely is not the only ERAD substrate of UBR4/5. Additionally our newly added figure 2E suggested that Hrd1 is also involved in HC degradation, therefore making HC

a less ideal substrate for UBR4/5 would perhaps increases the rate of HC degradation by other E3 ligases like Hrd1.
Additionally, while we agree that it would be interesting to test the degradation speed of HC with different first amino acid, we do not believe that it would be relevant to understanding the degradation of HC in our CHO system since using clinically tested and confirmed signal peptides and relevant first amino acid plays a critical role in expression of properly processed and expressed antibodies. As our CHO system is developed to express antibodies at very high titers and productivity rates, changing the framework signal peptide aa sequence would introduce many wrinkles for titer and product quality comparability criteria, which renders degradation rate comparisons invalid. For example, a change in first aa sequence can result in improper signal peptide cleavage or other product quality changes that in turn can change the HC flux within the ER, Golgi, and ultimately its secretion and degradation rates. Without extensively comparing and confirming effects of aa change in the vicinity of signal peptide (which is not in the scope of this study), it would be very difficult to draw clear conclusions from such studies.
The same is true about changing the pH in a production culture, which change many things, including transcription rates, post-translational modifications rates, and also antibody trafficking through the secretory pathways. Making it nearly impossible to understand which of these changes affect HC flux the most. Another wrinkle that pH change introduces is the requirement for bioreactors use, which are very expensive to operate and will not be approved by the management for this work (all our experiments are performed in shake flask).

3. The possibility that a protease cleaves the N-terminal half of the HC protein to simplify the clearing of ophan subunits is intriguing. However, based on multiple published studies, one would expect that the C-terminal "hinge-CH2-CH3" fragment, which should be folded and assembled into dimers, would then be secreted, while the VH-CH1 fragment would be targeted for degradation due to the unfolded CH1 domain. However, the data presented here do not include both the full-length protein and the C-terminal fragment on their blots of the media. Do the authors have an explanation as to why ERQC for the full-length γ heavy chain is so poor in this system?

We have included the full WB image as supplementary material below for the reviewer to view and in our data it is clear that in the media there is no cleaved HC fragment (in both HC only and HC/LC expressing cells) observed. Our CHO expression system including the cell lines and the cell culture media are actually very robust in assembly, folding and secretion of antibody molecules (making 2-10 grams/liter of antibodies in serum free media), therefore, not a large subset of HC molecules would be subjected to ERAD at a given time in these cells. Perhaps, the scenario that the reviewer explains could occur in a different expression system when the cell's ER is overwhelmed with unfolded HC molecules and proteolytic cleavage, but this does not apply to our CHO expression system.



4. PDIA3, aka ERp57, is the oxido-reductase associated with calnexin. Calnexin is not the primary chaperone for unassembled γ heavy chains, since the only stably unfolded domain in this protein should be the CH1 domain, which is not glycosylated. So even if PDIA3 has protease activity (the published literature on this possibility is very limited), it is surprising that it would be targeted to γ heavy chain. I recognize that the mutational analyses were meant to distinguish between isomerization and protease activity, but the same mutations used to inhibit proteinase activity should also inhibit reductase activity. Thus, it would be worthwhile to see if PDIA3 is acting to reduce the HC dimers in this system to aid in their extraction.

Qiu et al., 2004 had shown that ApoB100 is cleaved by PDIA3, and the cleavage generates a 50kDa band. Although the main function of PDIA3 is as a disulfide isomerase, it is highly possible that it also has hydrolytic activity. And its function as a hydrolytic enzyme may not need the chaperone of calnexin. And furthermore, the cleavage site is indeed in the CH1 domain, which is most likely unfolded and may recruit BiP and BiP binds the unfolded HC and keeps it in a conformation in which the cysteine residues are accessible for PDI. We have also shown that cysteine protease inhibitor inhibited HC extraction. Besides, as the folding of V-CH1 domain occurs and is stabilized by LC, the translation and folding of the rest of the HC molecule is not halted, the folding of the glycosylated CH2-CH3 domains occurs quicker than V-CH1 domains (as the reviewer pointed to in comment #3) and CH2-CH3 domains can engage calnexin as part of a glycosylated protein.

Other points:

Figure 2. What is the nature of the high molecular weight heavy chain species? It is about twice the size of the γ heavy chain Is this possibly a non-specific protein recognized by the anti-Flag antibody? The authors mention that the lines producing only HC are unstable and rapidly lose HC expression. Some of these chain-loss lines would make great controls for their experiments. All of the bands detected by anti-Flag should be part of a single image instead of shown as individual panels.

The high molecular species can be recognized by both anti-Flag and anti-human Fc antibodies, and the HMWS bands disappear when the samples are further treated with reducing SDS sample buffer containing 5% 2-Mercaptoethanol for extra 5 min. That is why we believe the upper HMWS is the dimer of HC. We have included a clarifying figure below to show that the HMWS bands can be further reduced to 55 kDa HC molecules for the reviewer to view (for both panels WB: anti-Flag).
The reason for showing the bands as individual panels is because sometimes the bands are far from each other and we want to save some space. Also, sometimes these bands have different intensities (since detecting antibody binds differentially to HC monomer or dimer), therefore bands could be overexposed or underexposed depending on exposure time. When shown in different panels both bands can be visualized clearly and at the relevant intensities.

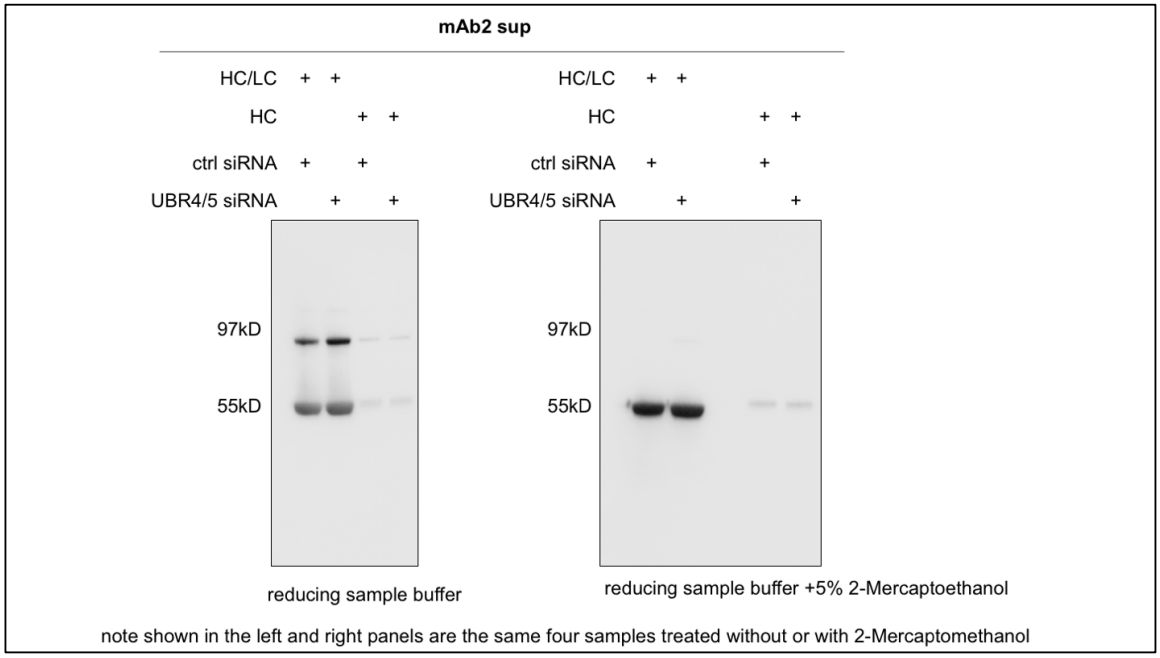
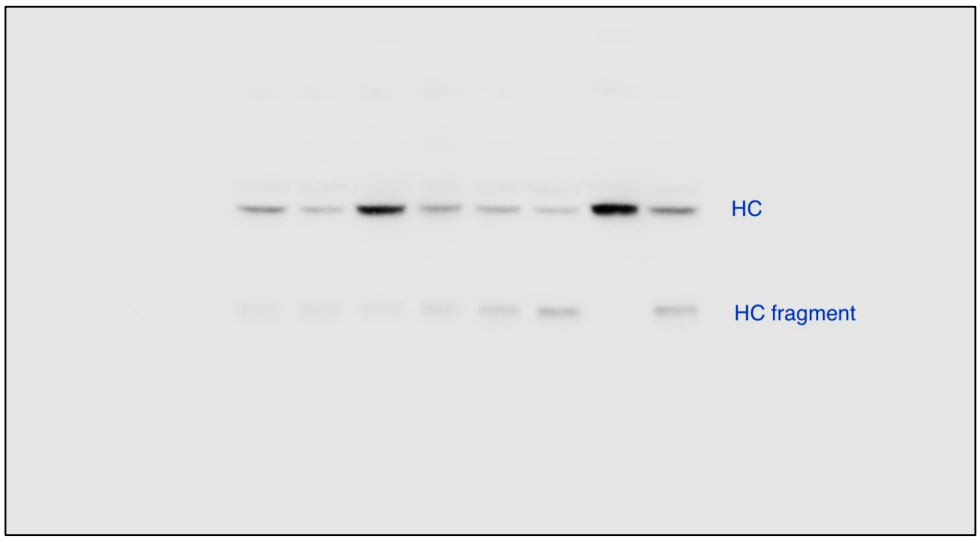


Figure 3. The single cleavage site very near the end of the CH1 domain is intriguing. Again, the full-length HC and HC fragment should be on a single blot instead of being shown as separate panels. Importantly, the CH1 domain is unstructured when unassembled. The authors also show similar amounts of this cleavage product in

cultures from cells expressing both HC and LC. Are these generated only from unassembled HC, or is the site in the CH1 domain cleaved even after assembly with LC? Based on the structure presented, it seems the authors might be implying this. However, the perceived lack of specificity between unassembled and assembled heavy chains for this protease are difficult to model into a role in ERQC.

It would be really difficult to differentiate unfolded HC and assembled HC in HC/LC expressing cells. But HC/LC expressing cells do have higher levels of HC secreted into the medium. It is possible that assembled HC/LC complexes are secreted while unassembled HC is cleaved and degraded. We have added this point in the text.
We have included a supplementary figure info that have all these HC species in a single image for the reviewer and the readers to view.



Figures 4E and F provide the only images in which the full-length HC and HC fragment are shown in a single panel. In 4E, both forms are present in near equal levels under control, steady-state conditions. This is surprising if the cleaved fragment is an intermediate in ERAD. Pulse-chase experiments would go a long way to prove precursor product relationship. However, in 4F there is very little of the fragment and nearly all of the HC is present as the high molecular weight form. Is it possible this high molecular weight band is non-specific? The ubiquitin signals should be normalized to the amount of HC for each treatment. Was this done in the presence of a proteasome inhibitor? There is nothing in the text or legend to indicate this. In Figure 4C and 4E, the fragment disappears when PDIA3 is knockdown alone, but large amounts of it are generated when both the E3s and PDIA3 are knocked down together. How do the authors reconcile this data if PDIA3 is the protease?

CHX chase assay in Supplementary Figure 3B and 5B showed that when protein synthesis was stopped, HC fragment level could still increase. This confirmed the fragment is generated by cleavage. It is less likely that a fragment is synthesized from the middle of a protein. The fragment/HC ratio is relatively high in this cell line because

mAb2 is a hard-to-express molecule, and may have folding problem. All the HCs that fold and assemble properly are secreted out of the cells very fast, only the HCs with folding problems remain in the ER and will be subjected to cleavage and degradation. Not all IgG molecules that we have tested generate the same level of HC fragment, and the difference is perhaps due to these HCs' expression levels as well as their ability to fold properly.

The Fig 4F is probed for Ub antibody, arrowheads indicate where HC and HC fragment should be localized on the gel. This point is added to the figure legends. Note that in this experiment we are showing the intracellular HC, and HC HMWS can only be detected in the media. Therefore the 97kD band in Fig 4F is likely to be a non-specific band and this assay is performed without MG132 treatment.

The last point is explained in the text, it is likely due to inefficient KD of PDIA3 and inability of cells to degrade the HC fragment generated by the remaining PDIA3 due to lack of (or reduced) UBR4 and UBR5 expression, as UBR4 and UBR5 were depleted in these cells. Please do note that PDIA3 is a more abundant protein compared to UBR4/5.

March 3, 2020

Re: JCB manuscript #201908087R

Dr. Shahram Misaghi
Genentech Inc.
1 DNA Way
south san francisco, CALIFORNIA 94080

Dear Dr. Misaghi,

Thank you for submitting your revised manuscript entitled "UBR4 and UBR5 together with Protease PDIA3 Mediate the ERAD of Unfolded Antibody Heavy Chains". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

For resubmission, please provide a response to Reviewer #1's concern regarding the lack of a significant effect on heavy chain turnover under conditions where PDIA3 is inhibited. This may be clarified with changes to the text to provide a more convincing explanation for the modest effect of PDIA3 inhibition, or by the use of an appropriate PDIA3 knockdown experiment.

please also attend to the following formatting requirements:

- Suggested alternative title to make the main advance accessible to as broad an audience as possible:
"UBR E3 ligases and the PDIA3 protease control degradation of unfolded antibody heavy chain by ERAD"
- Provide main and supplementary text as separate, editable .doc or .docx files
- Provide figures as separate, editable files according to the instructions for authors on JCB's website, paying particular attention to the guidelines for preparing images at sufficient resolution for screening and production
- Provide tables as excel files
- Add a paragraph after the Materials and Methods section briefly summarizing the online supplementary materials
- Add Acknowledgements section
- Add conflict of interest statement to Acknowledgements section
- State IRB that approved animal experiments in Materials and Methods

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

We requested the authors of this manuscript to provide three additional pieces of evidence to support their model that PDIA3 cleaves unfolded antibody heavy chains in order to make these heavy chains competent for ERAD.

The first request was for pulse chase or CHX turnover data to demonstrate that loss of UBR4/5 and PDIA3 specifically impair the turnover of heavy chain, as only steady state accumulation data was originally provided. These experiments were performed and added as Supplemental Figures 3C and 5B, respectively. Based on these data, it appears that loss of UBR4/5 impairs heavy chain turnover, but loss of PDIA3 (using a PDIA3 inhibitor) does not convincingly impair heavy chain turnover. This is an important point to clarify.

The second and third queries were sufficiently addressed by the authors.

Reviewer #2 (Comments to the Authors (Required)):

Although there are a few minor points that remain to be further clarified, considering the policy of JCB towards only one round of revisions, these revisions are satisfactory. I support this paper to be published in JCB.

Dear Editor,

Many thanks for your consideration to publish our manuscript “UBR4 and UBR5 together with Protease PDIA3 Mediate the ERAD of Unfolded Antibody Heavy Chains”. Here we have responded to Reviewer #1’s concern, hopefully to their and your satisfaction, and revised our manuscript accordingly. We have also changed the title, added Acknowledgement, conflict of interest statement and animal experiment approval statement (page 15) in the manuscript. We are now submitting the revised version of the manuscript together with 6 figures and 1 supplementary file. You may find all the relevant files on the website.

Thank you again for your consideration.

Sincerely,
Danming Tang

March 3, 2020

Re: JCB manuscript #201908087R

Dr. Shahram Misaghi
Genentech Inc.
1 DNA Way
south san francisco, CALIFORNIA 94080

Dear Dr. Misaghi,

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In the right panel of Supplementary Figure 5B, CHX and PDIA3 inhibitor pHMB were added at the same time to block both protein synthesis and HC cleavage. Adding pHMB significantly reduced the increase of HC fragment compared to the control condition (left panel). Also, adding pHMB caused a 1.4-fold reduction of the clearance rate of the full length HC compared to the control condition (the calculated degradation rates were added in Supplementary Figure 5B). These results suggest that PDIA3 is involved in HC cleavage, and HC cleavage helps to accelerate HC degradation. PDIA3 inhibition does not fully block degradation of the full length HC since these molecules can still be ubiquitinated by E3 ligases and degraded by the proteasome. But because the cleavage releases the folded C-terminal domain of the HC while allows the unfolded N-terminal domain to be extracted from ER, when PDIA3 is inhibited, HC degradation happens at a slower rate. Additionally, a 4-hour PDIA3 inhibitor treatment perhaps is not long enough to result in major clogging of a large number of degron channels to significantly impact HC degradation, as it was the case for the PDIA3 knockdown experiment (Figure 3C) when PDIA3 siRNAs were transfected for 4 days. That said, a short pHMB treatment during CHX chase experiments provides a better control since the starting materials of both the control and the PDIA3 inhibition conditions come from the same cultured cells (Supplementary Figure 5B).

We clarified this for the readers in page 25 of the manuscript.

The second and third queries were sufficiently addressed by the authors.

Reviewer #2 (Comments to the Authors (Required)):

Although there are a few minor points that remain to be further clarified, considering the policy of JCB towards only one round of revisions, these revisions are satisfactory. I support this paper to be published in JCB.