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The C-degron pathway eliminates mislocalized proteins and products of deubiquitinating enzymes

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1st Editorial Decision 30th Jul 2020

Thank you for submitting your manuscript on C-degron pathway characterization to The EMBO Journal. It has now been seen by three expert referees, whose comments are copied below. As you will see, all referees acknowledge the extensive data sets as well as the potential importance of your findings and conclusions, but they also retain a number of serious concerns that would need to be satisfactorily clarified before publication may be warranted. I realize that this will require substantive further time and efforts, but with all referees in principle being interested in seeing an improved version of this work, I would still like to give you an opportunity to address the reviewers' criticism via a major revision of this study. For such a revised manuscript to be successful, it will be crucial to better place the new results in the context of already published data (in particular from Koren et al), and to decisively address the issues with the GPS libraries and screens as stated most explicitly by referee 1 (as well as to generally improve the clarity and detail of the methodological descriptions, to allow better referee assessment). Furthermore, extensive new experiments may be needed to address the pervasive lack of statistical analysis and replicates.

Should you be able to adequately and fully address these points, as well as the various more specific comments by all three referees, we shall be happy to consider a revised manuscript further for EMBO Journal publication.

REFEREE REPORTS

Referee #1:

In this manuscript, Yeh, Huang et al. seek to understand the specificity and functions of C-degrons.

First, the authors used the ratiometric GPS reporter fused to random peptides to gain insights into what makes a C-degron, concluding that C-termini ending mostly with glycine or alanine residues acts as degrons, and that these signals can be modulated by other amino acids present near the C-terminus, e.g. another glycine residue at the second C-terminal position. Using bioinformatic analysis of various proteomes the authors show that such C-degrons are depleted at C-termini of eukaryotic proteins, especially in C-termini that locate in the cytosol and would therefore be accessible to the specific E3 ligases involved in their recognition. Yeh, Huang et al. then embark on a search for substrates of these C-degron pathway and suggest that mislocalized proteins, one example being the mitochondrial protein MIC19, and products of proteolysis by deubiquitinating enzymes can be substrates of C-degron pathways. Interestingly, mislocalized MIC19 appears to be targeted for degradation both via its C-degron and an N-degron. Finally, the authors show that although several viral proteins, specifically those of SARS-CoV, end with what look like C-degrons, their C-termini are an exception to the C-degron consensus and thus escape detection and degradation.

While some of the findings are potentially interesting, I find that the manuscript is nearly impossible to review.
First, the GPS assay used to dissect degrons is simplified to such an extent that the results of its use are uninterpretable (see point 1).
Second, the authors appear to ignore prior literature that details a substantial part of their conclusions (see point 2).
Third, throughout the manuscript there are statements and conclusions that are very imprecise or directly contradicted by the data in the figures; for many (almost all?) experiments only a single replicate is presented, making it impossible to draw conclusions; some experiments are described to such a superficial level that I was left guessing what was done; and the presentation of some types of experiments (for instance flow cytometry, recurrently) in the figures is in my opinion inappropriate (see point 3).
These three points are details below with a non-exhaustive list of examples.

1. In the GPS random peptide assay, random peptides are fused to a ratiometric reporter that provides information on the turnover of the fusion. Complex libraries of such reporters are introduced into cells followed by flow cytometry to determine the percentage of cells in the population that express unstable reporters (these cells should have low GFP/RFP ratios). The authors use this setup in Fig.1 to understand the determinants of different C-degrons.
However, despite the authors' statement, the percentage of cells with low GFP/RFP ratios is not indicative of the fraction of random peptides that act as degrons due to uneven representation of reporters in such libraries. Due to biases in synthesis of oligos used to construct such libraries, the frequency of different reporters in a pooled library can vary by 2-3 order of magnitude. Without sequencing of reporters present in the stable and unstable bins (as for instance done with illumina sequencing by Kats et al. 2018 using a similar reporter system in yeast PMID: 29727619, by Koren et al. 2018 PMID: 29779948 or by Timms et al. 2019 using the GPS reporter system in human cells PMID: 31273098), it is impossible to know how many and which reporters were stable or unstable. This makes in my opinion the experiments described in Fig.1 uninterpretable. Adding to it, in these experiments the authors sample a random subset of up to 20^12 possible variants but no information is provided about the number of cells in each library, complicating the interpretation of their results.
Furthermore, different libraries that likely sample different subsets of variants in this large 20^12 space are compared (e.g. Fig.1B or Fig.1D). This is not appropriate. Finally, some libraries are subsequently perturbed using e.g. shRNA constructs to inhibit specific BC-box proteins, but such perturbations are bound to reduce library complexity, for instance due to cell death upon viral

transduction. How do the authors control for such effects?

2. In figures 2 and 3, the authors examine various proteomes and proteins in different subcellular compartments for presence of C-degrons, observe depletion of proteins with glycine C-degrons from eukaryotic proteomes and conclude that C-degron-mediated degradation shapes eukaryotic protein evolution/selection. A substantial portion of this analysis was previously reported by Koren et al. 2018 PMID: 29779948. I find it remarkable that the authors do not contrast their findings to or even mention the results of Koren et al. 2018, despite citing this study in their introduction.

3. Issues with experimental design, data presentation and description, in order of appearance.

Throughout the manuscript, for most experiments only a single replicate is presented, making it difficult to draw conclusion.

Related to Fig.1, end of page 7 the authors state: "Nearly all stochastic peptides terminated with diGly or R__KG triggered KLHDC2 or KLHDC3-mediated degradation, respectively (Fig. 1D, 1E)." However, according to the figure, only ~40% of peptides terminated with diGly triggered KLHDC2-mediated degradation, and ~70% of peptides terminated with R__KG triggered KLHDC3-mediated degradation.
In addition, according to Fig.1D,E - ~95-100% of peptides terminated with diGly triggered degradation of the GPS reporter. Yet a similar experiment in Fig.7D shows only ~50-60% of peptides terminated with diGly triggered degradation of the GPS reporter. How do the authors reconcile this discrepancy?

The manuscript relies heavily on flow cytometry experiments. However, virtually no information is provided about the experimental setup, sample gating, data analysis (for example background correction). Almost all plots in the figures have no axis scales and, as stated by the authors in the methods section, were scaled in various ways and are thus not directly comparable. This is not appropriate. Moreover, in many plots that purport to show two samples in gray and dark gray only one is visible.

Fig.2D - shows some quantification but of which assay? How many replicates?

Fig.3B,F,G, 5G - the authors claim to measure protein stability. But instead of using their ratiometric GPS assay, the authors use luciferase or GFP reporters. These only allow quantifying protein abundance, not stability. In addition, the quantification of the luciferase assay in Fig.3B, reported as mean+-SD, has an unlikely SD of zero for two samples.

The experiments in Fig.4 are left essentially unexplained in the manuscript, including putative substrates such as BMP5 and BMP7 that don't behave as expected. But a strong conclusion is drawn on their basis "Thus, our findings have revealed that C-degron pathways eradicate misplaced cellular proteins caused by failed protein targeting."

All western blots are missing MW markers.

No scale bars on some microscopy figures (e.g. Fig. 5B,E).

Regrading Fig.5C the authors state "Nevertheless, blocking the C-degron pathway, either by inhibiting Cul2 or KLHDC2, resulted in trace amounts of MIC19 appearing in the cytosol (Fig. 5C), suggesting that mitochondrial transport of a proportion of endogenous MIC19 failed."

This is an important result to argue that MIC19 is an endogenous substrate of C-degron pathways as most other experiments in Fig.5 are performed with MIC19 mutants, truncations or in cells treated with myristoylation inhibitor, to force MIC19 mislocalization. It would be important to have quantification of multiple replicates to support the authors conclusion.

In addition, the authors should describe in the figure legends which antibodies are used for each experiment, both for immunofluorescence and Western blots. This is especially important for the HA-MIC19 proteins, where using an HA or MIC19 antibody is relevant for the interpretation of the results. Related to this, in the methods section, the authors mention that "We tried but failed to detect exogenous MIC19 signals using an HA antibody, so we used a MIC19 antibody instead. The signal from endogenous MIC19 was too low to be detected under the above-described staining conditions." It would be important to show this result as otherwise the experiments with HA-MIC19 cannot be interpreted.

In Fig.6C, is it unclear whether the authors express separately full length proteins and N-terminal domains (NTD) or, as in Fig. 6B, express full length proteins but detect NTD that result from processing by DUBs. Are these anti-GFP westerns or are the authors using antibodies against full length proteins? If these are 6 separate westerns, then there should be 6 separate loading controls, not 1.

4. Based on experiment in Fig.5 the authors conclude that MIC19 can be targeted for destruction by ZYG11B via an N-terminal glycine degron and by KLHDC2 via a C-terminal GG degron. This conclusion relies in part on mutants to the C-terminus of MIC19 that interfere with the C-degron under conditions that also impair MIC19 targeting to mitochondria. However, mutation to the MIC19 C-terminus could in principle also affect the CHCH domain and thus interfere with its localization to mitochondria. Can the authors rule out this possibility?

Minor points:

1. In figure 1F, G, it is not clear the criteria that authors use to determine if the effect of the distance between residues is 'high' or 'low'. While changes in the spacing between R and G in R_G reporters is considered to "mildly" affect degradation, changes in the spacing between R and G in R_G_ reporters is considered as an important factor. However, the observed relative % of degradation in the figure does not go in the same direction.

2. S1B: The conclusion claimed in the text "...we did identify a few diGly-ending peptides that did not trigger KLHDC2-mediated proteolysis...." is very imprecise. Which sequences specifically? Based on which statistical test?

3. Some figure panels are duplicated in different figures. Some images in figure S4H and figure 5J are the same. And some images in figure S3E and figure 3H are also the same. Ideally, duplication of panels should be avoided, but when this is not possible, this should be mentioned in the figure legends.

4. In figure 4F, the two Western blots are positioned as top and bottom, but the main text refers to them as left and right.

5. Figures 5B and S4B. How can the authors be sure that MIC19NT-GFP is located in the ER without any co-localization immunofluorescence with an ER protein?

6. The authors should list in the supplement all constructs used in the manuscript and include in the text or figure legends more details about the components of these constructs. Also, are all the constructs expressed using lentiviral transduction?

Referee #2:

Recent studies have revealed that a new class of eukaryotic 'degrons', sequence motifs that signal a protein's ubiquitination and subsequent degradation, occur at or close to the protein C-terminus. This manuscript by Yeh et al. extends earlier work that employed GPS-based assays to uncover details of the sequences that comprise these 'C-degrons' and the CRL2 E3 ligase subunits responsible for their recognition. Additionally, the work identifies physiological substrates of C-degron mediated degradation and provides important new insight into the functional significance of the C-degron pathway.

Overall, both the scope of this study and its technical quality make the work a good candidate for publication in EMBO J. However, some aspects of the paper were confusing and warrant fuller explanation or revisions. Also, some of the results should include statistical analyses. These issues are elaborated below.

1. In many of the figures (e.g., multiple panels in Fig. 1; Fig. 2D; Fig. 7C), the reproducibility of the data and the statistical significance of the differences that underlie critical conclusions are impossible to gauge. The authors need to provide measures of the reliability of their assays and, where appropriate, P values.

2. As mentioned by the authors on p. 7 (bottom), a strength of the approach that employs a random peptide library in GPS-based assays is that it can facilitate uncovering sequence variations that tune substrate degradation. The presentations of the results (e.g., see Fig 1F), however, highlight only select residues of the peptide sequences queried, the rest of the residues being notated as 'x'. Use of 'sequence logo' type presentations could provide more information; these could be provided as supplementary information.

3. What was the size of the peptide library used for the GPS analyses? For a given sequence, what is the expected redundancy (i.e., how many cells analyzed for each sequence)?

4. The results in Fig. 2C should be discussed more fully. It probably makes sense that Gly does not have a reduced frequency in bacterial protein C-termini, but the authors should comment about the reduced C-terminal Gly in yeast proteins; to my knowledge, there is no CRL2 homolog in yeast.

5. For the various protein stabilities determined in Fig. 2D, do their C-term sequences agree with the peptide-based results in Fig. 1F?

6. In Fig. 3B, the standard deviations for the values of the reporters without diGly seem impossibly small. Also, there's no reason to have these as a figure panel; they can be reported in the text.

7. Use of the GPS screen with membrane (e.g., ER or plasma membrane proteins) has the potential complication that the fused N-terminal GFP could perturb normal targeting or membrane insertion. This deserves comment.

8. (p.14, bottom) The claim that insufficient MIC19 protein caused mitochondria fragmentation is not well supported - the IMP-1088 treatment could have other effects. Further experiments would be needed if the authors want to make this point (e.g., effects of MIC19 knockdown and overexpression).

9. (p. 15 & 19) Proteolytic generation of C-terminal GlyGly does not necessarily involve DUB activity; instead, proteases that do not cleave ubiquitin conjugates could be responsible. The assumption the DUBs are used to expose GlyGly C-degrons needs to be corrected or, at a minimum, qualified.

10. (p. 17) The statement that the study demonstrates "...the dominance of protein "ends" in proteolysis-mediated protein quality inspection" is too sweeping. Perhaps "dominance" can be replaced by "importance".

11. Other wording is awkward, inappropriate, or grammatically incorrect:
- (p. 4) "normal" proteins; "illegitimate" C-termini; "have" yet to be achieved should be "has" yet to be achieved
- (p. 13) To say that MIC19-GFP "remained localized at the ER membrane" implies that it otherwise would transit to the cytoplasm; I don't think that was the authors' intention.
- (p. 17, bottom) What is meant by "unmediated causal relationship" is unclear
- (p. 19) I suggest dropping the prefix "neo" from N-degron and C-degron. Using the authors' logic, virtually all N-degrons should be renamed "neo-N-degron".

Referee #3:

In this manuscript, Yeh CW et al further characterize the C-end degron pathway and show it can particularly target proteins mislocalized in the cytosol for proteolysis. Notably, the authors further determined which residues in the C-end degron influence this degradation pathway using the GPS approach. Especially, they better defined the sequences recognized by the KLHDC3 and APPBP2 substrate adaptors. They then performed a series of computational analysis to show that C-terminal glycine residues are depleted across eukaryotes. Interestingly, this depletion is not seen for secreted and membrane proteins with an outward C-terminus. Therefore, the authors rationalize that these C-terminal degrons could have a prevalent role in the clearance of mislocalized proteins. Indeed, manipulations of C-termini or sequence targeting elements show that the C-end degrons can clear various aberrant proteins when localized in the cytosol. The authors then performed a series of elegant experiments with the mitochondrial protein MIC19 to further demonstrate how its C-end degron is important for clearance of the mislocalized protein and how it cooperates with a second N-end degron. MIC19 along other cleaved UBL domains were identified in a mass spectrometry experiment using KLHDC2 as a bait. These UBL domains were then shown to be cleared from the cell in a KLHDC2-dependent manner, showcasing another function for this pathway for clearing neo-C-end degrons. Curiously, coronaviruses, which also rely on the cleavage of the polyprotein using C-terminal GG residues, are resistant to the C-end degron pathway due to other stabilizing residues in the C-terminal region. Overall this is a very data dense paper with a broad array of results that are of good quality. One potential weakness is that the authors do not present evidence of a role for the C-end degron pathway in the clearance of mislocalized proteins in physiological conditions. For instance, does the deletion of KLDHC2 leads to the accumulation of secreted proteins in the cytosol when these proteins are not overexpressed or mutated? Nevertheless, the wide spectrum of results are assembled in a compelling story which is well suited

for publication in EMBO providing the authors can address several points.

It is unclear how the author assigned C-terminal sequences of genes. Among the first 10 proteins showcased in Figure 1H only 4 have a C-terminal GG based on the verified sequence in Uniprot (HIST1H4A, MBD3L2, MOCS2A, RPAP3). For CC2D2A, the isoform A (Q9P2K1, NP_001365544.1) is 1620 amino acid long and ends with IYVASLIRNR. The results of this experiment should be revised. The authors should repeat their computational analysis using Uniprot reviewed entries (20,353) and determine which isoforms are potentially included (or not) in the human database used for the computational analysis (36,644 entries). A complete list of cDNAs used in this study should be compiled with gene identifier and description of the used mutations/truncations. Unless I miss it, it is also unclear which constructs were used for DN Cul1-4 and which residues were omitted in the truncated secreted proteins (Fig 4B).

The authors should add a scale in most of their diagrams so that we can better compare results within a panel. For instance is HIST1H4A expressed at a much higher level than MBD3L2 in Figure 1H? The authors should also make sure that the same scale is used between two conditions. For example, in figure 3E, were overall Alg6 levels the same in CB-5083 treated cells compare to non-treated cells? If not, a scale should be added.

I don't find the evidence to support a role for VHL/p97 in the targeting of membrane proteins with added inward-facing C-degron compelling (as stated in page 10). In Figure 3D, the authors compare down regulation of VHL vs. KLHDC2. If VHL was required, then the degradation of Alg6 should be blocked. Perhaps, VHL is only properly inhibited here upon the addition of CB-5083 (after all VHL is fairly abundant and shRNA may not be sufficient for an efficient knock down). Alternatively, VHL is only required when KLHDC2 is over-expressed. One main issue is that it appears that VHL is also potentially used as a control in several experiments (Fig 1E, 3D, G, 4B-D). It would be important to show that VHL is significantly downregulated in these conditions (if not remove the data).

Overall, the authors should further clarify the choice of some their controls/targeted genes in the text (e.g. figure legend or method). In addition to selecting VHL, it won't be clear for the average reader why Fem1C (Fig 1E-G) and Zer1 (S4C) were selected.

I do not understand the rationale for linking the depletion of Gly -1 in mitochondrial proteins and a role for the C-degron in the clearance of mislocalized mitochondrial proteins. Do mitochondrial proteins with a C-terminal Glycine rely on atypical mitochondrial targeting? Or do they have a specific mitochondrial localization within the mitochondria? The authors should clarify which portion of membrane and mitochondrial proteins have a C-end degron using data from their previous analyses (Fig 1). Is the ensemble of these motifs enriched in these proteins? The p-values should also be corrected for multi testing in Figure 2 and related analyses.

In page 11, the authors should rephrase the statement that indicate that their "findings have revealed that C-degron pathways eradicate misplaced cellular proteins ..." The shown results (Fig 3 & 4) rely on artificial situations (e.g. overexpression, ablation of targeting sequence...) and therefore only reveal the 'potential' of this pathway. For instance, no assessed wildtype proteins accumulate in the cytosolic fraction in KHLDC2 or APPBP2 knock downs (Figure 4F). The authors should also disclose the fact that the mass spectrometry experiment exploit a condition in which cell compartmentalization is disrupted.

Minor points:

Could the author explain why they did not include KLHDC10 in their original assessment. This substrate adaptor was identified by the 2018 Cell paper from the Elledge group.

For Table 1, the ranking of the proteins in the list should be clarified (e.g. peptide number, sequence coverage…). FPR in the mass spectrometry analysis should be indicated in the method.

Figure 5K. Is there any evidence that shows the MTS cleavage of MIC19 occurs in the cytosol instead within the mitochondria? If not, the schematic should be revised.

Have the authors look at the 'stabilizing' residues in the C-terminal of SARS proteins to see whether they are also present in cytosolic protein with C-terminal GG residues.

Response to Referee 1

Referee #1:

In this manuscript, Yeh, Huang et al. seek to understand the specificity and functions of C-degrons. First, the authors used the ratiometric GPS reporter fused to random peptides to gain insights into what makes a C-degron, concluding that C-termini ending mostly with glycine or alanine residues acts as degrons, and that these signals can be modulated by other amino acids present near the C-terminus, e.g. another glycine residue at the second C-terminal position. Using bioinformatic analysis of various proteomes the authors show that such C-degrons are depleted at C-termini of eukaryotic proteins, especially in C-termini that locate in the cytosol and would therefore be accessible to the specific E3 ligases involved in their recognition. Yeh, Huang et al. then embark on a search for substrates of these C-degron pathway and suggest that mislocalized proteins, one example being the mitochondrial protein MIC19, and products of proteolysis by deubiquitinating enzymes can be substrates of C-degron pathways. Interestingly, mislocalized MIC19 appears to be targeted for degradation both via its C-degron and an N-degron. Finally, the authors show that although several viral proteins, specifically those of SARS-CoV, end with what look like C-degrons, their C-termini are an exception to the C-degron consensus and thus escape detection and degradation.

While some of the findings are potentially interesting, I find that the manuscript is nearly impossible to review.

First, the GPS assay used to dissect degrons is simplified to such an extent that the results of its use are uninterpretable (see point 1).

Second, the authors appear to ignore prior literature that details a substantial part of their conclusions (see point 2).

Third, throughout the manuscript there are statements and conclusions that are very imprecise or directly contradicted by the data in the figures; for many (almost all?) experiments only a single replicate is presented, making it impossible to draw conclusions; some experiments are described to such a superficial level that I was left guessing what was done; and the presentation of some types of experiments (for instance flow cytometry, recurrently) in the figures is in my opinion inappropriate (see point 3).

These three points are details below with a non-exhaustive list of examples.

1. In the GPS random peptide assay, random peptides are fused to a ratiometric reporter that provides information on the turnover of the fusion. Complex libraries of such reporters are introduced into cells followed by flow cytometry to determine the percentage of cells in the population that express unstable reporters (these cells should have low GFP/RFP ratios). The authors use this setup in Fig.1 to understand the determinants of different C-degrons. However, despite the authors' statement, the percentage of cells with low GFP/RFP ratios is not indicative of the fraction of random peptides that act as degrons due to uneven representation of reporters in such libraries. Due to biases in synthesis of oligos used to construct such libraries, the frequency of different reporters in a pooled library can vary by 2-3 order of magnitude. Without sequencing of reporters present in the stable and unstable bins (as for instance done with illumina sequencing by Kats et al. 2018 using a similar reporter system in yeast PMID: 29727619, by Koren et al. 2018 PMID: 29779948 or by Timms et al. 2019 using the GPS reporter system in human cells PMID: 31273098), it is impossible to know how many and which reporters were stable or unstable. This makes in my opinion the experiments described in Fig.1 uninterpretable. Adding to it, in these experiments the authors sample a random subset of up to 20^12 possible variants but no information is provided about the number of cells in each library, complicating the interpretation of their results. Furthermore, different libraries that likely sample different subsets of variants in this large 20^12 space are compared (e.g. Fig.1B or Fig.1D). This is not appropriate. Finally, some libraries are subsequently perturbed using e.g. shRNA constructs to inhibit specific BC-box proteins, but such perturbations are bound to reduce library complexity, for instance due to cell death upon viral transduction. How do the authors control for such effects?

Thank you for these points. We have never experienced uneven representation of reporters due to biases in oligo synthesis. Moreover, it is unfeasible to attain the 20∧12 space indicated by the referee. Even under an ideal (yet unrealistic) scenario (in which all variants are equally distributed and are only analyzed once), it would take 12,988 years to reach the 20∧12 space for one library by applying the maximum flow rate of 1×10^4 cells/sec for FACS analysis.

To fully address the referee's concerns regarding: 1) uneven library representation due to biases in oligo synthesis; 2) insufficient numbers of cell recordings to represent the library; and 3) treatment-induced reduction in library complexity, we have repeated the experiments in Fig. 1B-F **(new Fig. 1)** by constructing **multiple libraries for each of the examined C-terminal residues or motifs** (three and two libraries for Fig. 1B-D and Fig. 1E-F, respectively). Each library was generated from **"separately synthesized and purchased"** oligo libraries, which were used to construct independent GPS reporter cell libraries. **Each cell library separately treated with shRNAs was analyzed by FACS according to three random samplings with 100,000 cells each**. Consequently, nine and six replicates were performed for Fig. 1B-D and 1E-F, respectively, and the data are presented as mean ± standard deviation **(new Fig. 1B-1F)**. The small error bars shown in new Fig. 1 strongly support the reliability and robustness of our GPS random peptide platform.

Importantly, 10^5 cells far from cover all 20∧12 variants. Therefore, the small standard deviations from our multiple 10^5-sized random samplings further support that the features uncovered by our assay (such as benefits offered by positively-charged residues at the -2 position for the KLHDC3 degron) are truly independent of the identity of their neighboring residues. We have included information about library complexity and numbers of replicates and cells analyzed in the legend of Fig. 1 **(p.42, ln.26; p.43, ln.1-5)** or in our Materials and Methods **(p.25, ln.8-12, 18-21)**. We have also rewritten our text to make our rationale clearer **(p.5, ln.10-17; p.6, ln.8-16)**.

2. In figures 2 and 3, the authors examine various proteomes and proteins in different subcellular compartments for presence of C-degrons, observe depletion of proteins with glycine C-degrons from eukaryotic proteomes and conclude that C-degron-mediated degradation shapes eukaryotic protein evolution/selection. A substantial portion of this analysis was previously reported by Koren et al. 2018 PMID: 29779948. I find it remarkable that the authors do not contrast their findings to or even mention the results of Koren et al. 2018, despite citing this study in their introduction.

Koren *et al*. only analyzed functional proteomes, not aberrant proteins produced from synthesis errors. They also did not compare functional proteins in different cellular compartments. Importantly, they did not provide a direct connection between Gly/C-degron deficit and the avoidance of functional protein elimination.

We found that the Gly/C-degron shortfall is limited to functional proteins localized at cytosol, nucleus, mitochondria, or to membrane proteins having C-termini facing the cytosol, but not for secreted proteins or membrane proteins having their C-termini facing the extracellular space or ER lumen, suggesting that spatial quarantine protects proteins with C-degrons (Fig. 3A). Moreover, the Gly/C-degron deficit does not occur in abnormal proteins produced from translational frameshift or readthrough errors, products of NMD, or small peptides encoded by uORFs (Fig. 2B). We further show that mutating the extreme C-terminal residue of human proteins to Gly mostly triggered degradation, indicative of a direct causal

relationship between Gly ends and protein instability (Fig. 2D, 2E, Table EV3; original Fig 2D, 2E).

To address the referee's comment, we have revised our text to: *"It has been shown that C-degrons are depleted in functional eukaryotic proteomes (Koren et al., 2018), but whether this phenomenon is universal or specific to a subset of proteins remains unclear."* **(p.9, ln.9-11)**. We have also changed our subtitle to *"Gly/C-degron shortfall is limited to functional eukaryotic proteomes"* **(p.9, ln.8)**.

3. Issues with experimental design, data presentation and description, in order of appearance.

Throughout the manuscript, for most experiments only a single replicate is presented, making it difficult to draw conclusion.

We have repeated most of our experiments and now present the results as multiple replicates. Data in **new Fig. 1B-D, 1E-F, 7C** are presented as mean ± standard deviation from "nine", "six" or "three" replicates, respectively. We have also repeated our fractionation analysis of endogenous MIC19 twice and present it with quantification in **Fig. 5C and EV4C**. We have never before seen or been asked to display Western blots or FACS profilings as multiple replicates in figures. All of our Western blots were repeated multiple times to ensure reproducibility. Data in Fig. 7D are presented as histograms from analyzing 50,000 individual cells. For standard FACS analysis of cells carrying a single reporter, the histograms are derived from analyzing 20,000 individual cells (Fig. 1G, 2E, 3B-F, 4B-D, 5D, 6D, 7B, EV1B, EV2D, EV3C, EV4D-E, EV5B). We have now added information about the number of cells analyzed for GPS assay in our Materials and Methods **(p.24, ln.16-24)**.

Related to Fig.1, end of page 7 the authors state: "Nearly all stochastic peptides terminated with diGly or R__KG triggered KLHDC2 or KLHDC3-mediated degradation, respectively (Fig. 1D, 1E)." However, according to the figure, only ~40% of peptides terminated with diGly triggered KLHDC2-mediated degradation, and ~70% of peptides terminated with R__KG triggered KLHDC3-mediated degradation.

Indeed, ~23% of the degradation triggered by diGly- or R__KG-terminated random peptide libraries is KLHDC2/3-independent, i.e. the percentage degradation triggered by random 12-mers (X_{12}) is ~23% (the first column in new Fig. 1B and 1D (original Fig 1D and 1E)). Adding terminal diGly or R__KG enhances the percentage degradation to ~100% (Fig. 1B; original Fig. 1D). KLHDC3 knockdown reverses the additional 77% degradation triggered by R__KG to the background level (23%), so "ALL" degradation stimulated by the R__KG motif is KLHDC3-mediated (Fig. 1D; original Fig. 1E). We were unable to completely inhibit the activity of KLHDC2 by shRNA-mediated knockdown because the binding affinity between KLHDC2 and diGly degrons is exceptionally high (Kd ~3.75 nM for the SELK diGly degron; *Mol. Cell* 72, 813). Therefore, shKLHDC2 treatment only partially inhibited the

percentage degradation promoted by the diGly motif to ~60% (Fig. 1D; original Fig. 1E).

In response to the referee's concern, we have removed the term "nearly all" from our text and revised our statement to: *"Stochastic peptides terminated with diGly or R__KG effectively triggered KLHDC2 or KLHDC3-mediated GFP degradation, respectively (Table EV1, Fig. 1B, 1D)."* **(p.8, ln.21-23)** In addition, we now explain why shRNA-mediated KLHDC2 knockdown only partially inhibited KLHDC2 activity on **p.7, ln.12-15**.

In addition, according to Fig.1D,E - ~95-100% of peptides terminated with diGly triggered degradation of the GPS reporter. Yet a similar experiment in Fig.7D shows only ~50-60% of peptides terminated with diGly triggered degradation of the GPS reporter. How do the authors reconcile this discrepancy?

Data in Fig. 1B, 1D (original Fig. 1D-E) and Fig. 7D cannot be directly compared. The percentage degradation shown in Fig. 1B and 1D has been calibrated by removing the signals contributed from truncated peptides due to random insertion of internal TAG stop codons (the calibration procedure is explained on p.25, ln.21-24 and p.26, ln.1-7 (original p.23, ln.6-16). In contrast, in Fig. 7D we display global stability profilings of libraries without calibrations. Fig. 7D is presented to demonstrate that the diGly upstream sequence "RELN" from SARS-CoV NSP1 inhibits KLHDC2-mediated degradation.

The manuscript relies heavily on flow cytometry experiments. However, virtually no information is provided about the experimental setup, sample gating, data analysis (for example background correction). Almost all plots in the figures have no axis scales and, as stated by the authors in the methods section, were scaled in various ways and are thus not directly comparable. This is not appropriate. Moreover, in many plots that purport to show two samples in gray and dark gray only one is visible.

1. We have now added detailed information to the revised Materials and Methods **(p.24, ln.16-24)** about our flow cytometry experiments including machine settings, sample gating, and data analysis. Note, we did not apply background correction during data analysis.
2. The GFP/RFP histograms exported from the BD FACSDiva™ software are displayed by default as a 'linear' scale. Consequently, the signal range for optimal presentation is narrow. Since stability varied dramatically among proteins, we often observed "off-scaling" (i.e. when the GFP/RFP value is too close to or beyond the maximum value of the axis scale) when presenting the stability of multiple proteins with distinct stabilities using the same ratio scaling. To solve this problem, we recorded multiple scalings for GFP/RFP for each sample and presented the one with optimum resolution (different ratio scaling is "analogous" to different exposure time for Western blotting). Therefore, we mentioned in our original manuscript: *"To enhance the resolution of the GPS results, we conducted multiple scaling for the GFP/RFP ratio. Since stability*

varied dramatically among proteins, the plots were scale-adjusted for optimal resolution. The GFP/RFP ratios from separate plots cannot be compared directly" (original p.22, ln.12-16). Similarly, it is inappropriate to compare the intensity of protein bands from different Western blots developed for different exposure times.

To address the referee's comment, we consulted flow cytometry experts and have now converted the GFP/RFP histograms into "log" scale using FlowJo software. We have now included axis scales in all GPS plots **(Fig. 1G, 2E, 3B-D, 4B-D, 6D, 7B-D, EV1B, EV2D, EV3C, EV5B)**.

3. We have adjusted the transparency of the dark gray coloration to make both samples more visible **(Fig. 1G, 3B-D, 5D, 6D, 7B, 7D, EV1B, EV3C, EV4E, EV5B)**.

Fig.2D - shows some quantification but of which assay? How many replicates?

The protein stability assay presented in original Fig. 2D represents an analysis of 20,000 individual GPS reporter cells for each protein (total 72 proteins, or 36 protein pairs). Instead of displaying 36 individual GPS plots that would occupy too much space, we only present the relative mean stability (GFP/RFP) of wild-type and mutant proteins.

To improve data interpretation, we now display our results both as a simplified graph **(new Fig. 2D)** and an extended view table that provides protein names and detailed numbers **(Table EV3)**.

Fig.3B,F,G, 5G - the authors claim to measure protein stability. But instead of using their ratiometric GPS assay, the authors use luciferase or GFP reporters. These only allow quantifying protein abundance, not stability. In addition, the quantification of the luciferase assay in Fig.3B, reported as mean+-SD, has an unlikely SD of zero for two samples.

1. **Original Fig. 3B (now moved to main text):** We have rephrased our statement to *"…., **supporting** the notion of localization-restrictive degradation"*. In addition, we have deleted original Fig. 3B, and instead describe our results in the text (as suggested by referee #2) **(p.11, ln.2-6)**.

2. **Fig. 3E and 3F (original Fig. 3F, 3G)**: To emphasize that we measured protein "abundance" not "stability", we have rewritten our statement to: *"We compared the **abundance** of these mitochondrial GFP-degron fusion proteins with and without functional C-degron pathways (Fig. 3E, 3F). Supporting the notion of localization-selective degradation, the degree of difference was inversely correlated with ……. "* **(p.12, ln.2-6)**.

3. **Fig. 5G:** In fact, we do not claim to measure protein stability for Fig. 5G. The goal of Fig. 5G is to document the degree of MIC19 mis-localization by comparing relative MIC19 protein abundance in cytosol and mitochondria.

4. For **original Fig. 3B**, we performed the experiment in triplicate and used the SpectraMax® Paradigm® Multi-Mode Microplate Detection Platform from Molecular Devices LLC to measure luciferase activity. This device only reports to two decimal places (i.e. x.xxE+xx). Thus, standard deviations below 0.01 are not reported. For clarity, we now present those standard deviations as <0.01 **(p.11, ln.2-6)**.

   We provide the original data below for your reference.

	#1	#2	#3	Mean	STDEV
Fluc	5.69E+06	5.69E+06	5.69E+06	**5.69E+06**	**<1.00E-02**
Fluc-GG	1.18E+04	1.32E+04	1.04E+04	**1.18E+04**	**1.38E+03**
Gluc	3.00E+07	3.00E+07	3.00E+07	**3.00E+07**	**<1.00E-02**
Gluc-GG	1.47E+07	1.50E+07	1.50E+07	**1.49E+07**	**1.73E+05**

The experiments in Fig.4 are left essentially unexplained in the manuscript, including putative substrates such as BMP5 and BMP7 that don't behave as expected. But a strong conclusion is drawn on their basis "Thus, our findings have revealed that C-degron pathways eradicate misplaced cellular proteins caused by failed protein targeting."

We respectfully disagree with the referee on this point. In fact, our data strongly suggest that BMP5 and BMP7 are indeed *bona fide* APPBP2 substrates. Although the effect of APPBP2 knockdown on the stability of BMP5 and BMP7 is modest when compared to other proteins by GPS assay, this modest stabilization disappears when the Gly of their C-degrons is deleted (ΔG, Fig. 4C), strongly indicative of its specificity. Importantly, we show that overexpression of APPBP2 **"significantly**" destabilized wild-type BMP5 and BMP7, but not their C-degron mutants (ΔG) (Fig. 4D). Moreover, signal peptide-deleted mutant BMP5 and BMP7 (ΔSP) accumulated in the cytosol when APPBP2 was inhibited (Fig. 4G).

In response to the referee's concern, we have added new statements about BMP5 and BMP7 on **p.12, ln.20-24 and p.13, ln.1**: *"Although APPBP2 knockdown only modestly stabilized BMP5 and BMP7, this effect was not observed for their C-degron mutants (ΔG) (Fig. 4C). In addition, BMP5 and BMP7, but not their C-degron mutants, were significantly destabilized when the abundance of APPBP2 was elevated (Fig. 4D). Moreover, signal peptide-deleted mutant BMP5 and BMP7 (ΔSP) accumulated in the cytosol when APPBP2 was inhibited (Fig. 4G)."* We have also rephrased our statement to: *"...our findings suggest that C-degron pathways **potentially** eradicate misplaced cellular proteins caused by failed protein targeting"* **(p.13, ln.3-5)**.

All western blots are missing MW markers.

Apologies, we have now added MW markers to all Western blots **(Fig. 4E-G, 5C, 5G, 6B-C, 6E, EV4C, EV5A)**.

No scale bars on some microscopy figures (e.g. Fig. 5B,E).

Apologies, we have now added scale bars to **Fig. 5B, 5E, EV4B, EV4F and EV4G**.

Regrading Fig.5C the authors state "Nevertheless, blocking the C-degron pathway, either by inhibiting Cul2 or KLHDC2, resulted in trace amounts of MIC19 appearing in the cytosol (Fig. 5C), suggesting that mitochondrial transport of a proportion of endogenous MIC19 failed."
This is an important result to argue that MIC19 is an endogenous substrate of C-degron pathways as most other experiments in Fig.5 are performed with MIC19 mutants, truncations or in cells treated with myristoylation inhibitor, to force MIC19 mislocalization. It would be important to have quantification of multiple replicates to support the authors conclusion.

We have repeated the endogenous MIC19 fractionation experiment and now present data and quantification from two replicates in **Fig. 5C and EV4C**.

In addition, the authors should describe in the figure legends which antibodies are used for each experiment, both for immunofluorescence and Western blots. This is especially important for the HA-MIC19 proteins, where using an HA or MIC19 antibody is relevant for the interpretation of the results. Related to this, in the methods section, the authors mention that "We tried but failed to detect exogenous MIC19 signals using an HA antibody, so we used a MIC19 antibody instead. The signal from endogenous MIC19 was too low to be detected under the above-described staining conditions." It would be important to show this result as otherwise the experiments with HA-MIC19 cannot be interpreted.

1. Based on the author guidelines for EMBO Journal (https://www.embopress.org/page/journal/14602075/authorguide), we describe the specific antibodies we used in our Materials and Methods **(p.27, ln.11-16; p.28, ln.3-4)** (original p.24, ln.21-23; p.25, ln.1-2, 14).
2. We now include the control confocal images of U2OS cells stained with a MIC19 antibody, as requested **(Fig. EV4H)**. In addition, we now mention in the main text that: "*We used a MIC19 antibody to detect exogenously expressed HA-MIC19 because the signal from HA antibodies was too weak. We confirmed that levels of endogenous MIC19 were too low to be detected under this staining condition (Fig. EV4H).*" **(p.15, ln.9-12)**.

In Fig.6C, is it unclear whether the authors express separately full length proteins and N-terminal domains (NTD) or, as in Fig. 6B, express full length proteins but detect NTD that result from processing by DUBs. Are these anti-GFP westerns or are the authors using antibodies against full length proteins? If these are 6 separate westerns, then there should be 6 separate loading controls, not 1.

As in Fig. 6B, we expressed GFP-tagged full-length proteins and detected NTDs processed by Dubs. We chose to separately display full-length and NTD proteins because the optimum exposure times to detect them by Western blotting differ (due to their distinct protein abundances).

To address the referee's comment, we now show full-length and NTD proteins on the same blots, as well as separate loading controls for each protein examined **(new Fig. 6C)**.

4. Based on experiment in Fig.5 the authors conclude that MIC19 can be targeted for destruction by ZYG11B via an N-terminal glycine degron and by KLHDC2 via a C-terminal GG degron. This conclusion relies in part on mutants to the C-terminus of MIC19 that interfere with the C-degron under conditions that also impair MIC19 targeting to mitochondria. However, mutation to the MIC19 C-terminus could in principle also affect the CHCH domain and thus interfere with its localization to mitochondria. Can the authors rule out this possibility?

Yes, we can rule out that scenario. We performed two independent sets of experiments to draw the above-mentioned conclusion. Apart from mutating MIC19 to specifically inhibit N- or C-degron-mediated proteolysis (*in cis*; as mentioned by the referee), we also examined the effect of ZYG11B and KLHDC2 knockdown on **"wild-type"** MIC19 (*in trans*; **Fig. 5I, 5J, EV4J**). We obtained consistent conclusions from both these approaches.

Minor points:

1. In figure 1F, G, it is not clear the criteria that authors use to determine if the effect of the distance between residues is 'high' or 'low'. While changes in the spacing between R and G in R_G reporters is considered to "mildly" affect degradation, changes in the spacing between R and G in R_G_ reporters is considered as an important factor. However, the observed relative % of degradation in the figure does not go in the same direction.

As shown in Fig. 1E (bottom four rows), R__KG motifs with different spacings all trigger KLHDC3-mediated degradation so we conclude that the spacing between R and G in R__KG motifs only "mildly" affects degradation. In contrast, as shown in Fig. 1F right top, only RxxGx effectively promotes APPBP2-dependent degradation (fourth row from top), though the number of residues downstream of Gly can range from one to three (rows 2-4 from top). Changing the R-G spacing of R_G_ reporters (rows 5-11) cancels APPBP2-mediated degradation, i.e. the relative percentage degradation with or without APPBP2 are all close to 1. Therefore, the spacing between R and G in the R_G_ reporters is crucial. We have now added row numbers to our explanations for clarity **(p.8, ln.5-9)**.

2. S1B: The conclusion claimed in the text "...we did identify a few diGly-ending peptides that did not trigger KLHDC2-mediated proteolysis...." is very imprecise. Which sequences specifically? Based on which statistical test?

We wanted to emphasize that C-terminal diGly alone does not guarantee KLHDC2-mediated degradation. As shown in Table EV1 (original Fig. S1B), Fig. 7C and 7D, the upstream sequences of diGly affect or even inhibit KLHDC2-mediated

degradation. The sequences of diGly peptides and their potency in triggering KLHDC2-dependent degradation are shown in Table EV1 (original Fig. S1B).

To avoid confusion, we have now deleted the statement "…we did identify a few diGly-ending peptides….". In addition, we have replaced Fig. S1B with an extended view table **(Table EV1)**. Protein stabilities were measured by analyzing 20,000 individual GPS reporter cells.

3. Some figure panels are duplicated in different figures. Some images in figure S4H and figure 5J are the same. And some images in figure S3E and figure 3H are also the same. Ideally, duplication of panels should be avoided, but when this is not possible, this should be mentioned in the figure legends.

We feel that showing the complete datasets with all necessary controls and placing them side-by-side for comparison would greatly facilitate readers interpreting our conclusions. Nevertheless, displaying them all as formal figures would occupy too much space. In response to the referee's concern, we now mention in the figure legends of Fig. 3G (original 3H), 5E and 5J **(p.45, ln.14-15; p.47, ln.11-12, 23-24)** that complete image sets are provided in Fig. EV3F, EV4F and EV4J, respectively.

4. In figure 4F, the two Western blots are positioned as top and bottom, but the main text refers to them as left and right.

Apologies, now corrected **(p.46, ln.5-7)**.

5. Figures 5B and S4B. How can the authors be sure that MIC19NT-GFP is located in the ER without any co-localization immunofluorescence with an ER protein?

We have now rephrased our statement to: *"Consistently, the localization of GFP N-terminally tagged with the 24-residue N-terminal myristoylation motif of MIC19 (MIC19^{NT})* ***resembled*** *that of an ER membrane protein (Fig. 5B, S4B)"* **(p.13, ln.20-22)**.

6. The authors should list in the supplement all constructs used in the manuscript and include in the text or figure legends more details about the components of these constructs. Also, are all the constructs expressed using lentiviral transduction?

We have now added new **Table EV5** containing information on all constructs and cDNAs used in this manuscript. We also mention that: *"All exogenous proteins and shRNAs were expressed via lentivirus-mediated transduction."* on **p.23, ln.23 and p.24, ln.1**.

Response to Referee 2

Referee #2:

Recent studies have revealed that a new class of eukaryotic 'degrons', sequence motifs that signal a protein's ubiquitination and subsequent degradation, occur at or close to the protein C-terminus. This manuscript by Yeh et al. extends earlier work that employed GPS-based assays to uncover details of the sequences that comprise these 'C-degrons' and the CRL2 E3 ligase subunits responsible for their recognition. Additionally, the work identifies physiological substrates of C-degron mediated degradation and provides important new insight into the functional significance of the C-degron pathway.

Overall, both the scope of this study and its technical quality make the work a good candidate for publication in EMBO J. However, some aspects of the paper were confusing and warrant fuller explanation or revisions. Also, some of the results should include statistical analyses. These issues are elaborated below.

1. In many of the figures (e.g., multiple panels in Fig. 1; Fig. 2D; Fig. 7C), the reproducibility of the data and the statistical significance of the differences that underlie critical conclusions are impossible to gauge. The authors need to provide measures of the reliability of their assays and, where appropriate, P values.

Fig. 1B-G, 7C: We have repeated our experiments and now present data as mean ± standard deviation from multiple replicates **(new Fig. 1B-F and 7C)**. Nine and six replicates were performed for Fig. 1B-D and 1E-F, respectively. The resulting small error bars support the reliability of our random peptide platform. We have also now added p values in **Fig. 1B** to show the significance of the ability of terminal Gly or Ala to stimulate degradation.

Fig. 2D: Protein stability, as presented in original Fig. 2D, was measured by analyzing 20,000 individual GPS reporter cells for each protein (total 72 proteins or 32 protein pairs). We only showed the relative mean stability (GFP/RFP) of wild-type and mutant proteins rather than displaying 36 individual GPS plots that would occupy too much space. To improve our data presentation, we have reconfigured Fig. 2D to display our results as a simplified graph **(new Fig. 2D)** and now provide a new extended view table **(Table EV3)** that provides detailed protein information and numbers.

Fig. 2B, 2C, 3A and Table EV1: We have now corrected our p values by FDR (false discovery rate) for multi-testing.

2. As mentioned by the authors on p. 7 (bottom), a strength of the approach that employs a random peptide library in GPS-based assays is that it can facilitate uncovering sequence variations that tune substrate degradation. The presentations of the results (e.g., see Fig 1F), however, highlight only select residues of the peptide sequences queried, the rest of the residues being notated as 'x'. Use of 'sequence logo' type presentations could provide more information; these could be provided as supplementary information.

We feel that our results are not suitable for presentation in the “sequence logo” format proposed by the referee since that format is a graphic representation of

sequence conservation. A sequence logo is created from a collection of aligned sequences and depicts the consensus and diversity of the sequences. Rather than being a conventional sequence comparison, our GPS random peptide platform "adds-in" a targeted feature under **"randomized" sequence backgrounds (i.e. x)** and examines the effect of that feature across a heterogeneous pool. Thus, apart from the amino acid we purposely specify at a defined position, the remaining residues (i.e. x) are mixtures of stochastic amino acids. To improve clarity, we have now added more detailed descriptions about our GPS random peptide platform on **p.6, ln.8-16**.

3. What was the size of the peptide library used for the GPS analyses? For a given sequence, what is the expected redundancy (i.e., how many cells analyzed for each sequence)?

The complexity of our peptide library is approximately **1×10^{10}**. It is unfeasible to reach the 20^12 space (4.096×10^{15}; 20 amino aids at 12 positions) covering all possible variants for 12-residue peptides using current molecular and cell biology technologies. Even if we possessed the ideal library comprising all possible variants at equal frequency and each variant was only analyzed once, it would still take 12,988 years to reach the 20∧12 space for one library given a current maximum flow rate of 1×10^{4} cells/sec for FACS analysis.

In order to prevent distortion caused by: 1) uneven library representation due to biases in oligo synthesis; 2) insufficient number of cell recordings to represent the library; and 3) treatment-induced reduction in library complexity, we constructed **multiple libraries for each of the examined C-terminal residues or motifs** (three and two libraries for Fig. 1B-D and Fig. 1E-F, respectively). Each library was generated from "separately synthesized and purchased" oligo libraries, which were used to construct independent GPS reporter cell libraries. Each cell library was analyzed by FACS according to **three random samplings of 100,000 cells each**. Consequently, **nine and six replicates were performed for Fig. 1B-D and 1E-F**, respectively, and the data are presented as mean ± standard deviation **(new Fig. 1B-1F)**. The small error bars shown in new Fig. 1 strongly support the reliability of our GPS random peptide platform.

Importantly, 10^5 cells far from cover all 20∧12 variants. Therefore, the small standard deviations from multiple 10^5-sized random samplings further support that the features uncovered by our assay (such as benefits offered by positively-charged residues at the -2 position of the KLHDC3 degron) are truly independent of the identity of neighboring residues. We have now included information about library complexity and numbers of replicates and cells analyzed in the legend of Fig. 1 **(p.42, ln.26; p.43, ln.1-5)** or in the Materials and Methods **(p.25, ln.8-12, 18-21)**. We have also revised our text to make our rationale clearer to readers **(p.5, ln.10-17; p.6, ln.8-16)**.

4. The results in Fig. 2C should be discussed more fully. It probably makes sense that Gly does not have a reduced frequency in bacterial protein C-termini, but the authors should comment about the reduced C-terminal Gly in yeast proteins; to my knowledge, there is no CRL2 homolog in yeast.

The referee is correct about there not being a CRL2 homolog in yeast. It is possible that the Gly/C-degron pathway does not exist in yeast. Alternatively, the yeast C-degron pathway may involve another member of the CRL ubiquitin ligase family (e.g. CRL1 or CRL3). For instance, a Cul4 homolog also does not exist in yeast. The stability of XPC/Rad4 protein is regulated by CRL4A in human, but by CRL3 in yeast (*EMBO J.* 25: 2529; *Acta Biochim Biophys Sin* 43: 919).

To address the referee's comment, we have added the following statement: *"It is unclear why terminal Gly is depleted in the yeast proteome given that a CRL2 homolog does not exist in yeast."* on **p.19, ln.18-20**.

5. For the various protein stabilities determined in Fig. 2D, do their C-term sequences agree with the peptide-based results in Fig. 1F?

We do not expect the Gly^{-1}-dependent protein stabilities determined in Fig. 2D to agree with the results in Fig. 1F because the experiment in Fig. 1F specifically targets KLHDC3-mediated degradation. There are multiple KLHDC family members involved in the Gly/C-degron pathway; for example, KLHDC2, KLHDC3 and KLHDC10 target GG, R__G, and [W/P/A]G, respectively. Moreover, we show that the R__KG motif does not guarantee KLHDC3-mediated degradation, i.e. functional proteins harboring terminal R__KG are not eliminated by KLHDC3 (Fig. EV1B). Mechanisms such as structural masking may prevent functional proteins from being eliminated by the C-degron pathway.

6. In Fig. 3B, the standard deviations for the values of the reporters without diGly seem impossibly small. Also, there's no reason to have these as a figure panel; they can be reported in the text.

Agreed. We have now deleted Fig. 3B and instead describe our results in the text as suggested **(p.11, ln.2-6)**. We performed this experiment in triplicate and used the SpectraMax® Paradigm® Multi-Mode Microplate Detection Platform from Molecular Devices LLC to measure luciferase activity. This device only reports to two decimal places (i.e. x.xxE+xx). Thus, standard deviations below 0.01 are not reported. For clarity, we now present those standard deviations as <0.01 **(p.11, ln.2-6)**.

We provide the original data below for your reference.

	#1	#2	#3	Mean	STDEV
Fluc	5.69E+06	5.69E+06	5.69E+06	**5.69E+06**	**<1.00E-02**
Fluc-GG	1.18E+04	1.32E+04	1.04E+04	**1.18E+04**	**1.38E+03**
Gluc	3.00E+07	3.00E+07	3.00E+07	**3.00E+07**	**<1.00E-02**
Gluc-GG	1.47E+07	1.50E+07	1.50E+07	**1.49E+07**	**1.73E+05**

7. Use of the GPS screen with membrane (e.g., ER or plasma membrane proteins) has the potential complication that the fused N-terminal GFP could perturb normal targeting or membrane insertion. This deserves comment.

N-terminal GFP tagging only affects the membrane insertion of type I single-pass transmembrane proteins that rely on their N-terminal signal peptide for membrane anchoring. All membrane proteins examined in Fig. 3 (3B-3D, original 3C-3E) are multi-pass transmembrane proteins whose membrane anchoring relies on their internal transmembrane domains. Accordingly, their localization should not be perturbed by N-terminal GFP.

To address the referee's concern, we have added a **new Fig. EV3D** showing the localization of GFP-tagged membrane proteins examined in Fig. 3B-C. We have also added the sentence: *"N-terminal GFP tagging does not affect membrane anchoring of these transmembrane proteins."* on **p.11, ln.9-10**.

8. (p.14, bottom) The claim that insufficient MIC19 protein caused mitochondria fragmentation is not well supported - the IMP-1088 treatment could have other effects. Further experiments would be needed if the authors want to make this point (e.g., effects of MIC19 knockdown and overexpression).

The function of MIC19 in crista integrity and mitochondria function has been characterized and reported previously (*J Biol Chem* 287: 39480), as cited in our manuscript. In our main text **(p.13, ln.12-15;** original **p.11, ln.23-24)**, we mentioned that: *"MIC19 is a mitochondrial inner membrane protein crucial for crista integrity and mitochondrial function (Darshi et al, 2011). Downregulation of MIC19 causes fragmented mitochondria, restricted oxygen consumption and glycolysis, and reduced growth rate."*

9. (p. 15 & 19) Proteolytic generation of C-terminal GlyGly does not necessarily involve DUB activity; instead, proteases that do not cleave ubiquitin conjugates could be responsible. The assumption the DUBs are used to expose GlyGly C-degrons needs to be corrected or, at a minimum, qualified.

Indeed, proteolytic generation of C-terminal diGly does not necessarily involve Dubs activity. For instance, the coronaviral protease PLpros cleaves at NSP protein junctions following a diGly motif. To address the referee's comment, we now specify in the text that: *"Collectively, these data suggest that the C-degron^{KLHDC2} pathway can eliminate diGly-ending cleavage products of Dubs* ***or other proteases.***" and *"The C-degron^{KLHDC2} pathway may also eliminate diGly-terminated products of* ***other proteases besides Dubs.***" on **p.18, ln.1-2 and p.21, ln.5-7**, respectively.

10. (p. 17) The statement that the study demonstrates "...the dominance of protein "ends" in proteolysis-mediated protein quality inspection" is too sweeping. Perhaps "dominance" can be replaced by "importance".

We have changed “dominance” to “importance” **(p.19, ln.9)**.

11. Other wording is awkward, inappropriate, or grammatically incorrect:
- (p. 4) "normal" proteins; "illegitimate" C-termini; "have" yet to be achieved should be "has" yet to be achieved.

We have rephrased “normal”, “illegitimate” and “have” to “functional”, “deviant” and “has”, respectively **(p.4)**.

- (p. 13) To say that MIC19-GFP "remained localized at the ER membrane" implies that it otherwise would transit to the cytoplasm; I don't think that was the authors' intention.

Apologies. We have deleted the word “remained” **(p.15, ln.2)**.

- (p. 17, bottom) What is meant by "unmediated causal relationship" is unclear.

We have changed to “**direct** causal relationship” **(p.19, ln.23)**.

- (p. 19) I suggest dropping the prefix "neo" from N-degron and C-degron. Using the authors' logic, virtually all N-degrons should be renamed "neo-N-degron".

Agreed. We have deleted the prefix “neo” **(p.21, ln.10-11)**.

Response to Referee 3

Referee #3:

In this manuscript, Yeh CW et al further characterize the C-end degron pathway and show it can particularly target proteins mislocalized in the cytosol for proteolysis. Notably, the authors further determined which residues in the C-end degron influence this degradation pathway using the GPS approach. Especially, they better defined the sequences recognized by the KLHDC3 and APPBP2 substrate adaptors. They then performed a series of computational analysis to show that C-terminal glycine residues are depleted across eukaryotes. Interestingly, this depletion is not seen for secreted and membrane proteins with an outward C-terminus. Therefore, the authors rationalize that these C-terminal degrons could have a prevalent role in the clearance of mislocalized proteins. Indeed, manipulations of C-termini or sequence targeting elements show that the C-end degrons can clear various aberrant proteins when localized in the cytosol. The authors then performed a series of elegant experiments with the mitochondrial protein MIC19 to further demonstrate how its C-end degron is important for clearance of the mislocalized protein and how it cooperates with a second N-end degron. MIC19 along other cleaved UBL domains were identified in a mass spectrometry experiment using KLHDC2 as a bait. These UBL domains were then shown to be cleared from the cell in a KLHDC2-dependent manner, showcasing another function for this pathway for clearing neo-C-end

degrons. Curiously, coronaviruses, which also rely on the cleavage of the polyprotein using C-terminal GG residues, are resistant to the C-end degron pathway due to other stabilizing residues in the C-terminal region. Overall this is a very data dense paper with a broad array of results that are of good quality. One potential weakness is that the authors do not present evidence of a role for the C-end degron pathway in the clearance of mislocalized proteins in physiological conditions. For instance, does the deletion of KLDHC2 leads to the accumulation of secreted proteins in the cytosol when these proteins are not overexpressed or mutated? Nevertheless, the wide spectrum of results are assembled in a compelling story which is well suited for publication in EMBO providing the authors can address several points.

We very much appreciate this referee's positive response. In fact, we do present evidence of a role for the C-degron pathway in the clearance of mislocalized endogenous MIC19 under physiological conditions **(Fig. 5C, EV4C)**.

It is unclear how the author assigned C-terminal sequences of genes. Among the first 10 proteins showcased in Figure 1H only 4 have a C-terminal GG based on the verified sequence in Uniprot (HIST1H4A, MBD3L2, MOCS2A, RPAP3). For CC2D2A, the isoform A (Q9P2K1, NP_001365544.1) is 1620 amino acid long and ends with IYVASLIRNR. The results of this experiment should be revised. The authors should repeat their computational analysis using Uniprot reviewed entries (20,353) and determine which isoforms are potentially included (or not) in the human database used for the computational analysis (36,644 entries). A complete list of cDNAs used in this study should be compiled with gene identifier and description of the used mutations/truncations. Unless I miss it, it is also unclear which constructs were used for DN Cul1-4 and which residues were omitted in the truncated secreted proteins (Fig 4B).

1. We assigned the C-terminal sequences of proteins based on the NCBI database. For CC2D2A, isoform c/4 (NM_003538.3; NP_001158192.1) is 111 amino acids long and ends with VCNPSTLEGR**GG**. We now list in **new Table EV5** for reference the gene IDs, mRNA and protein accessions, and the sequences of the proteins examined to generate Fig. 1G (original Fig. 1H).
2. We have repeated our computational analyses using Uniprot-reviewed entries (20,353) and now display the results in **new Fig. EV2A, EV2B and Table EV2.** The conclusions we derive from analyzing sequences from both databases (i.e. Uniprot and NCBI) are essentially the same.
3. We have added a **new Table EV5** containing all constructs and cDNAs used in this manuscript (including DN Cul1-4).
4. The proteins examined to generate Fig. 4B are "full-length" and N-terminally tagged with GFP. Since signal peptides and MTSs (mitochondrial targeting sequences) both occur at protein N-termini and they need to be exposed to be functional, all tested proteins in Fig. 4B are mislocalized.

The authors should add a scale in most of their diagrams so that we can better compare results within a panel. For instance is HIST1H4A expressed at a much higher level than MBD3L2 in Figure 1H? The authors should also make sure that the same scale is used between two conditions. For example, in figure 3E, were overall Alg6 levels the same in CB-5083 treated cells compare to non-treated cells? If not, a scale should be added.

We have now included axis scales in all GPS plots **(Fig. 1G, 2E, 3B-D, 4B-D, 6D, 7B, 7D, EV1B, EV2D, EV3C, EV5B).** The GFP/RFP histograms exported from the BD FACSDiva™ software are displayed by default as a 'linear' scale. Consequently, the signal range for optimal presentation is narrow. Since stability varied dramatically among proteins, we often observed "off-scaling" (i.e. when the GFP/RFP value is too close to or beyond the maximum value of the axis scale) when presenting the stability of multiple proteins with distinct stabilities using the same ratio scaling. To solve this problem, we recorded multiple scalings for GFP/RFP for each sample and presented the one with optimum resolution (different ratio scaling is "analogous" to different exposure time for Western blotting). To address the referee's comment, we consulted flow cytometry experts and have now converted the GFP/RFP histograms into "log" scale using FlowJo software.

I don't find the evidence to support a role for VHL/p97 in the targeting of membrane proteins with added inward-facing C-degron compelling (as stated in page 10). In Figure 3D, the authors compare down regulation of VHL vs. KLHDC2. If VHL was required, then the degradation of Alg6 should be blocked. Perhaps, VHL is only properly inhibited here upon the addition of CB-5083 (after all VHL is fairly abundant and shRNA may not be sufficient for an efficient knock down). Alternatively, VHL is only required when KLHDC2 is over-expressed. One main issue is that it appears that VHL is also potentially used as a control in several experiments (Fig 1E, 3D, G, 4B-D). It would be important to show that VHL is significantly downregulated in these conditions (if not remove the data).

It seems that the referee has confused VHL with VCP/p97. VHL is a BC-box protein (a substrate receptor for CRL2 ubiquitin ligase), whereas VCP/p97 is an ATPase that has been shown to bind and extract ubiquitinated proteins from membranes or cellular structures to facilitate proteasome-mediated degradation (*Mol. Cell* 69: 182). We used VHL as an unrelated BC-box protein control for specificity (Fig. 1D, 3C, 3F and 4B-D, original Fig. 1E, 3D, 3G and 4B-D). Separately, we examined if the VCP/p97 ATPase is required for C-degron^{KLHDC2} pathway-mediated membrane protein degradation by treating cells with the VCP/p97 inhibitor CB-5083 (Fig. 3D; original Fig. 3E). To make this point clearer, we have now added the statement *"VHL and FEM1C serve as unrelated BC-box protein controls."* **(p.43, ln.10-11)**, and provide a new reference about VCP/p97 **(p.11, ln.14)**.

Overall, the authors should further clarify the choice of some their controls/targeted genes in the text (e.g. figure legend or method). In addition to selecting VHL, it won't be clear for the average reader why Fem1C (Fig 1E-G) and Zer1 (S4C) were selected.

Acknowledged. We have now added the following notes in the figure legends of Fig. 1D and EV4D (original S4C), respectively: *"VHL and FEM1C serve as unrelated BC-box protein controls."* **(p.43, ln.10-11)**, and *"ZYG11B and ZER1 are BC-box proteins involved in Gly/N-degron recognition (Timms et al. 2019)"* **(p.51, ln.20-22)**. We found that only ZYG11B can mediate the degradation of MIC19 (Fig. EV4D).

I do not understand the rationale for linking the depletion of Gly -1 in mitochondrial proteins and a role for the C-degron in the clearance of mislocalized mitochondrial proteins. Do mitochondrial proteins with a C-terminal Glycine rely on atypical mitochondrial targeting? Or do they have a specific mitochondrial localization within the mitochondria? The authors should clarify which portion of membrane and mitochondrial proteins have a C-end degron using data from their previous analyses (Fig 1). Is the ensemble of these motifs enriched in these proteins? The p-values should also be corrected for multi testing in Figure 2 and related analyses.

1. Mitochondrial proteins are synthesized in the "cytosol" and then "post-translationally" delivered into the mitochondria. Therefore, mitochondrial proteins harboring Gly/C-degrons will be cleared by the C-degron pathway if they mislocalize to cytosol. Similar to cytosolic and nuclear proteins, Gly^{-1} is depleted in mitochondrial proteins in order to escape Gly/C-degron-mediated proteolysis (Fig. 3A).
2. Gly^{-1} is depleted in mitochondrial proteins regardless of their mitochondria-targeting mechanisms. Most mitochondrial proteins enter mitochondria *via* their N-terminal MTS (mitochondria targeting sequence). MIC19 is a very rare exception that relies on its N-terminal Gly myristoylation and C-terminal CHCH domain to enter mitochondria.
3. Since for functional proteins, having C-degrons does not guarantee degradation (Fig. 1G, EV1B), we do not think it is appropriate to predict if a membrane or mitochondrial protein is regulated by the C-degron pathway solely based on their C-terminal sequences (i.e. the motifs characterized in Fig. 1).
4. Terminal Gly are "depleted" for mitochondrial proteins and membrane proteins having their C-terminal facing the cytosol (Fig. 3A).
5. We have now corrected our p-values by FDR (false discovery rate) for multi-testing **(Fig. 2B, 2C, 3A, EV2C, Table EV2)**.

In page 11, the authors should rephrase the statement that indicate that their "findings have revealed that C-degron pathways eradicate misplaced cellular proteins ..." The shown results (Fig 3 & 4) rely on artificial situations (e.g. overexpression, ablation of targeting sequence...) and therefore only reveal the 'potential' of this pathway. For instance, no assessed wildtype proteins accumulate in the cytosolic fraction in KHLDC2 or APPBP2 knock downs (Figure 4F). The authors should also disclose the fact that the mass spectrometry experiment exploit a condition in which cell compartmentalization is disrupted.

1. In fact, we do reveal that **"endogenous wild-type"** MIC19 accumulates in cytosol upon KLHDC2 or CRL2 inhibition **(Fig. 5C, EV4C)**. However, we have now rephrased our statement to: *"…our findings suggest that C-degron pathways **potentially** eradicate misplaced cellular proteins caused by failed protein targeting"* **(p.13, ln.3-5)**.
2. We have added the following sentence on **p.29, ln.4-5**: *"Cell compartmentalization was disrupted under this lysis condition"*.

Minor points:

Could the author explain why they did not include KLHDC10 in their original assessment. This substrate adaptor was identified by the 2018 Cell paper from the Elledge group.

The current manuscript is an extension of our earlier work (Lin *et al.*, 2018). We did not identify KLHDC10 substrates, so we did not focus on that BC-box protein.

For Table 1, the ranking of the proteins in the list should be clarified (e.g. peptide number, sequence coverage...). FPR in the mass spectrometry analysis should be indicated in the method.

1. We have rearranged the proteins in Table 1 based on alphabetical order, which is now explained in a footnote.
2. We now include FDR in the mass spectrometry analysis on **p.30, ln.19-20**. Peptides were identified with FDR < 1%.

Figure 5K. Is there any evidence that shows the MTS cleavage of MIC19 occurs in the cytosol instead within the mitochondria? If not, the schematic should be revised.

MIC19 does not have a MTS. Instead, MIC19 enters mitochondria *via* Gly^2 myristoylation at the cytosol. As stated on **p13, ln.17-20** (original p.12, ln.3-6), *"Unlike canonical mitochondrial proteins that rely on MTSs, Gly^2 myristoylation-mediated membrane-targeting and the CHCH domain contribute to the mitochondrial localization of MIC19 (Darshi et al, 2012; Uneda et al, 2019)".*

Have the authors look at the 'stabilizing' residues in the C-terminal of SARS proteins to see whether they are also present in cytosolic protein with C-terminal GG residues.

We have tried, but we couldn't establish a simple rule to explain the "stabilizing" effect. We will collaborate in future with structural biologists to address this issue.

Thank you for submitting your revised manuscript to The EMBO Journal. All three original reviewers have now looked at it again, and found their key criticisms generally satisfactorily addressed. Referees 1 and 3 still retain several minor/specific concerns, as you will see from the comments below, which I would ask you to respond to and (as appropriate) incorporate during a final round of revision.

During the final revision round, I would kindly ask you to also take care of the following editorial points.

REFEREE REPORTS

Referee #1:

The authors have substantially revised the manuscript, which is now in my opinion significantly improved. However, in my opinion a few points are not adequately addressed as detailed below:

1. Composition of GPS peptide libraries.
The authors clarify the complexity of the peptide libraries and performed replicates of the experiments in Fig.1. The issue is that stating that 10^5 cells were used in each experiment is not enough to judge the number of assayed peptides. Clearly it has be less than 10^5 peptides, but how much less?

2. Statements regarding importance of Cul2 and different adaptors.
I am puzzled by the authors argumentation that all R__KG degrons are recognized by KLHDC3, most/all -GG degrons are recognized by KLHDC2, most/all G degrons are recognized by CRL2, while the data shows that to be not the case. And I think the conclusions in this respect are overstated. For instance, in Fig.1C only about half of construct ending with XXXXG peptides are stabilized by expressing a dominant negative Cul2. That the % of peptides not affected by DN Cul2 is by chance similar to the % of degrons in the random X12 library is completely irrelevant. So the statement that "Gly-dependent degradation was mediated by the CRL2 ubiquitin ligase" is imprecise. In half of the cases, Gly-dependent degradation was mediated by the CRL2 ubiquitin ligase. In the other half, remains to be determined. The same applies to R__KG degrons vs KLHDC3 and -GG degrons vs KLHDC2. For instance, the new statement that "Stochastic peptides terminated with diGly (...) effectively triggered KLHDC2-mediated GFP degradation" is extremely ambiguous. The results in Fig. 1D show that to be the case for ~35% of -GG peptides. So one could just as easily conclude that stochastic peptides terminated with diGly do not effectively trigger KLHDC2-mediated GFP

degradation. The fact that complete depletion of KLHDC2 was not possible does not allow the conclusion that all/most -GG peptides would be KLHDC2-dependent degrons.

3. Flow cytometry - single cells are not replicates.
The authors substantially improved the description and presentation of flow cytometry experiments. However, the issue with the lack of replicates remains. That the histograms are derived from 20k or 50k cells does not deter from the fact that these are measurements of a single replicate.

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Building upon the work of Koren et al. 2018, the authors show that there is a depletion of -G and -GG C-termini from the human proteome but not from what the author call abnormal proteins, i.e. those that could be potentially generated by premature stop codons, frameshifts, and stop codon readthrough. I think it would be important to clarify in the main text that, in the authors bioinformatic analysis, abnormal proteins generated by frameshifts and stop codon readthrough are hypothetical proteins. Second, I think the terms "NMD proteins" or "products of nonsense-mediated decay" are incorrect. If I understand the authors correctly, what they actual refer to are truncated proteins that result from premature stop codons and the corresponding transcript variants have been annotated as NMD variants in bioMart. Perhaps refer to these as truncated proteins. Referring to these abnormal proteins as products of NMD implies that somehow nonsense-mediated mRNA degradation generates abnormal proteins, which is obviously not the case.

5. Description of experiments in Fig. 4. The authors now describe the experiments performed with BMP5 and BMP7 and their interpretation in detail. I would suggest doing the same with the rest of the data in this figure, which is clearly valuable.

Referee #2:

The authors' detailed rebuttal and revisions to their manuscript have satisfied the concerns outlined in my original critique.

Referee #3:

This is a revised manuscript from Yeh at al. on the characterization of C-degron. The authors have addressed most of my concerns and there are just a few remaining minor issues that should be fixed prior to publication

Figure 1. The authors should more precisely define what they mean by "random sampling" in 1B and replicates in 1C, D, E and F. Are these replicates derived from a combination of biological and technical replicates? They cannot combine biological and technical replicates to produce standard deviations. Biological replicates show sample variance while technical replicates show systematic noise/error. To convolute the two is incorrect. They should average the values from technical replicates and only show standard deviations from biological replicates.

Based on the presented data in Figure 1F, the authors cannot conclude that the negative charges are detrimental (middle of page 8). For this claim the authors should show there is a loss of turnover,

whereas the assay shows that the negative charge don't affect APPBP2-dependent degradation.

The new representation of the data for Figure 2D does not allow to claim that most mutations reduce stability (page 10). At most 7-8 candidates appear to be affected.

Scales in Figure 3C, D and F are not readable and should be adjusted accordingly

Response to Referees

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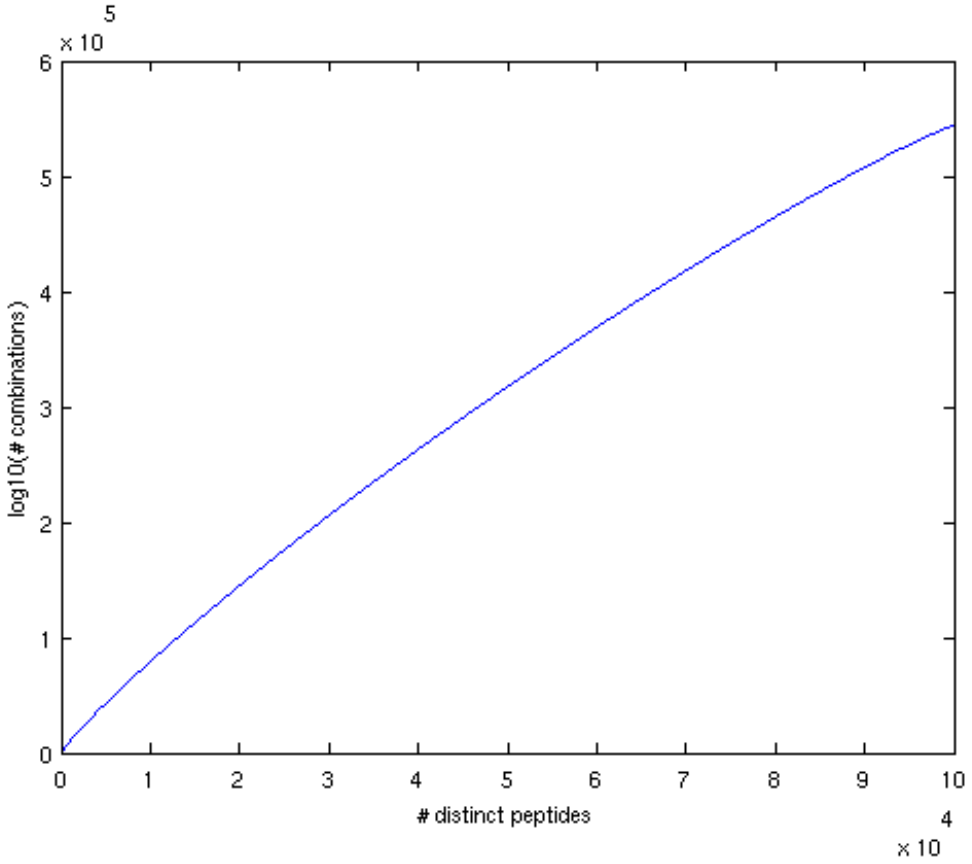
The complexity/variation of each random peptide library is approximately 10^{10} **(p.25, ln.14-15)**, and the size of random samples is 10^5. The number of distinct peptides drawn from random samples is approximated by the formula below:

Note, $N = 10^{10}$ represents the total number of distinct peptides and $n = 10^5$ is the number of samples randomly drawn from the peptides with replacement. Also, $P_n^N(k)$ represents the probability that the random samples contain k distinct peptides. $P_n^N(k)$ is proportional to the number of combinations of k distinct peptides retrieved from n random samples. By using Stirling's formula to approximate $\binom{N}{k}$, below we display log10 of the number of combinations with

varying k. $P_n^N(k)$ peaks at $k = n$ (all n peptides are distinct) and declines rapidly for smaller k values. For instance, $\frac{P_n^N(k=n)}{P_n^N(k=n-10)} \approx 3.6 \times 10^6$, which means the probability of retrieving ≥ 10 repeated peptides is negligible. The probability of retrieving ≥ 3 repeated peptides is not negligible: $\frac{P_n^N(k=n)}{P_n^N(k=n-3)} \approx 6$. Thus, we can be almost certain that the random samples are virtually all distinct.



2. Statements regarding importance of Cul2 and different adaptors. I am puzzled by the authors argumentation that all R__KG degrons are recognized by KLHDC3, most/all -GG degrons are recognized by KLHDC2, most/all G degrons are recognized by CRL2, while the data shows that to be not the case. And I think the conclusions in this respect are overstated. For instance, in Fig.1C only about half of construct ending with XXXXG peptides are stabilized by expressing a dominant negative Cul2. That the % of peptides not affected by DN Cul2 is by chance similar to the % of degrons in the random X12 library is completely irrelevant. So the statement that "Gly-dependent degradation was mediated by the CRL2 ubiquitin ligase" is imprecise. In half of the cases, Gly-dependent degradation was mediated by the CRL2 ubiquitin ligase. In the other half, remains to be determined. The same applies to R__KG degrons vs KLHDC3 and -GG degrons vs KLHDC2. For instance, the new statement that "Stochastic peptides terminated with diGly (...) effectively triggered KLHDC2-mediated GFP degradation" is extremely ambiguous. The results in Fig. 1D show

that to be the case for ~35% of -GG peptides. So one could just as easily conclude that stochastic peptides terminated with diGly do not effectively trigger KLHDC2-mediated GFP degradation. The fact that complete depletion of KLHDC2 was not possible does not allow the conclusion that all/most -GG peptides would be KLHDC2-dependent degrons.

We respectfully disagree with the referee on this point. We **do NOT** claim that *"all R__KG degrons are recognized by KLHDC3, most/all -GG degrons are recognized by KLHDC2, most/all G degrons are recognized by CRL2"*. Instead, we state that *"Gly-dependent degradation was mediated by CRL2 ubiquitin ligase, consistent with CRL2 playing a dominant role in C-degron pathways."* **(p.7, ln.4-6)**, *"More than 95% of diGly-ending random peptides stimulated degradation, whereas capping diGly with Leu blocked degradation, suggesting that exposure of the terminal diGly motif alone is typically adequate for KLHDC2 degron recognition."* **(p.7, ln.13-16)**, and *"All random peptides terminating with the R_KG motif promoted degradation and the R_KG motif-stimulated degradation is KLHDC3-mediated."* **(p.8, ln.6-8)**.

The implications that ***'Gly-dependent degradation was mediated by CRL2 ubiquitin ligase'*** (our statement) and *that of* ***'most/all G degrons are recognized by CRL2'*** (Referee's statement) are inherently different. Similarly, the underlying meanings of ***'the R_KG motif-stimulated degradation is KLHDC3-mediated'*** (our statement) and ***'all R__KG degrons are recognized by KLHDC3'*** (Referee's statement) are also very distinct. As shown in Fig. 1C and 1D, we found that 20~25% of peptides ending in random amino acids promoted degradation. Notably, peptides ending specifically with Gly or R_KG promoted degradation to ~55% or ~100%, respectively. Importantly, the **additional "Gly- or R_KG- dependent"** degradation was completely inhibited when the activity of CRL2 or KLHDC3 was respectively blocked (i.e. from 55% or 100% back to 20~25%). Therefore, we concluded that Gly-dependent or R_KG-stimulated degradation was mediated by CRL2 or KLHDC3, respectively. The same scenario applied to the diGly degron.

3. Flow cytometry - single cells are not replicates. The authors substantially improved the description and presentation of flow cytometry experiments. However, the issue with the lack of replicates remains. That the histograms are derived from 20k or 50k cells does not deter from the fact that these are measurements of a single replicate.

We agree with the referee that single cells cannot be considered replicates. We wish to emphasize that all experiments shown in our manuscript have been repeated for reproducibility and that we have presented one of our replicates as a representative example. Displaying flow cytometry data from a single replicate as a histogram is common practice in published peer-reviewed manuscripts.

4. Depletion of -G and -GG from functional vs abnormal proteins. Building upon the work of Koren et al. 2018, the authors show that there is a depletion of -G and -GG C-termini from the human proteome but not from what the author call abnormal proteins, i.e. those that could be potentially generated

by premature stop codons, frameshifts, and stop codon readthrough. I think it would be important to clarify in the main text that, in the authors bioinformatic analysis, abnormal proteins generated by frameshifts and stop codon readthrough are hypothetical proteins. Second, I think the terms "NMD proteins" or "products of nonsense-mediated decay" are incorrect. If I understand the authors correctly, what they actual refer to are truncated proteins that result from premature stop codons and the corresponding transcript variants have been annotated as NMD variants in bioMart. Perhaps refer to these as truncated proteins. Referring to these abnormal proteins as products of NMD implies that somehow nonsense-mediated mRNA degradation generates abnormal proteins, which is obviously not the case.

As clearly stated in our text and the subtitle *"Gly/C-degron shortfall is limited to functional eukaryotic proteomes"* **(p.9, ln.12)**, the goal of our bioinformatics analysis is to examine whether C-degron depletion is specific to functional proteomes. The expression of abnormal proteins due to frameshifts or stop codon readthrough is not the subject of our study. Importantly, we respectfully disagree with the referee's assertion that *"abnormal proteins generated by frameshifts and stop codon readthrough* ***are hypothetical proteins****"*. In fact, expression of these aberrant proteins has been clearly validated experimentally (*J Biol Chem 290: 28428; J Biol Chem RA120.014253; Nature 399: 776; Nature 441: 603*). Recent ribosome-profiling experiments have revealed widespread stop codon readthrough (i.e. upon 3'UTRs being protected by ribosomes) (*PLoS Genet 15: e1008141*). Frameshifting may be caused by genetic frameshift mutations or ribosomal frameshifting (translational frameshifting). Genetic frameshift mutations are caused by insertions or deletions of a number of nucleotides in DNA sequences that are not divisible by three (due to the triplet nature of the genetic codon). Ribosomal frameshifting is affected by the secondary structure of mRNA and can be programed (*Prog Nucleic Acid Res Mol Biol 64: 131; Science Advances 6:eaaz6969; J Biol Chem RA120.014253*). Ribosome collisions and overcompaction of polysomes are additional mechanisms by which ribosomal frameshifting can be triggered (*Cell Reports 28: 1679; PNAS 116: 21769*). Detection of aberrant transcripts and activation of NMD require the first round of translation, so truncated proteins are produced from NMD transcripts (*Annu. Rev. Gent 49: 339*).

Indeed, we mean truncated proteins produced from NMD transcripts. To address the referee's concern, we have revised our statement to *"abnormal proteins produced from ……,* ***NMD (nonsense-mediated mRNA decay) transcripts****, or ….."* **(p.9, ln.24; p.10, ln.1)**.

5. Description of experiments in Fig. 4. The authors now describe the experiments performed with BMP5 and BMP7 and their interpretation in detail. I would suggest doing the same with the rest of the data in this figure, which is clearly valuable.

In fact, we have already described these experiments in detail. As shown in **p.12, ln.20-24** and **p.13, ln.1**, *"Abolition of the predestined localization of those proteins,*

either by N-terminal capping (Fig. 4B, 4C, 4D, 4E, 4F top) or targeted peptide deletion (Fig. 4F bottom, 4G), specifically stimulated C-degron pathway-dependent protein destruction. Changing the C-termini of these proteins by either deletion or masking completely inhibited C-degron pathway-mediated degradation (Fig. 4C)." The C-terminal sequences and additional information of these proteins have also been detailed in Fig. 4A and Table EV5. In total, we characterized fifteen secretory or mitochondrial proteins that when mislocalized were eliminated by C-degron pathways. We applied the same approach to examine all of these proteins and our conclusions are consistent (Fig. 4B-G). Importantly, our goal is to demonstrate the physiological function of C-degron pathways in protein spatial quality control, but not to emphasize individual proteins that are regulated by C-degron pathways. Therefore, describing the experiments for each individual protein separately would be redundant and unnecessary.

Referee #2:

The authors' detailed rebuttal and revisions to their manuscript have satisfied the concerns outlined in my original critique.

We thank Referee #2 for his/her valuable suggestions to improve our manuscript.

Referee #3:

This is a revised manuscript from Yeh at al. on the characterization of C-degron. The authors have addressed most of my concerns and there are just a few remaining minor issues that should be fixed prior to publication

Figure 1. The authors should more precisely define what they mean by "random sampling" in 1B and replicates in 1C, D, E and F. Are these replicates derived from a combination of biological and technical replicates? They cannot combine biological and technical replicates to produce standard deviations. Biological replicates show sample variance while technical replicates show systematic noise/error. To convolute the two is incorrect. They should average the values from technical replicates and only show standard deviations from biological replicates.

The standard deviations indicated in Fig. 1B-F are derived solely from biological replicates. Based on the explanation in *Nature Methods* (https://www.nature.com/articles/nmeth.3091), *"**Biological replicates** are parallel measurements of **biologically distinct samples** that capture random biological variation, which may itself be a subject of study or a noise source. **Technical replicates** are repeated measurements of **the same sample** that represent independent measures of the random noise associated with protocols or equipment."* Since the sample space of all 12-residue peptides (20^X, where X is the number of random amino acids and ranges between 5 and 12) far exceeds the size of random sampling (10^5), the probability of retrieving repeated peptides in random samples is extremely low. Thus those random samples are expected to carry distinct

peptides and should be considered as biological replicates. In response to the referee's concern, we have now added a sentence to the legend of Fig. 1 **(p.43, ln.7-11)** to explain why those random samplings represent "biological replicates".

Based on the presented data in Figure 1F, the authors cannot conclude that the negative charges are detrimental (middle of page 8). For this claim the authors should show there is a loss of turnover, whereas the assay shows that the negative charge don't affect APPBP2-dependent degradation.

The relative % degradation with/without (+/-) APPBP2 equal to "1" means that stability with or without APPBP2 activity is the same, i.e. the assayed C-terminal motif does not trigger APPBP2-mediated degradation. As shown in Fig. 1F (right panel, row 4 from top), the relative % degradation +/- APPBP2 from the _R_G_ motif alone (xxxxxxxRxxGx) is ~1.8. Adding negative charges decreased the relative % degradation +/- APPBP2 to ~1 (Fig. 1F, left panel, row 6~13 from bottom). Therefore, we concluded that negative charges are detrimental.

The new representation of the data for Figure 2D does not allow to claim that most mutations reduce stability (page 10). At most 7-8 candidates appear to be affected.

The data shown in current Fig. 2D and Table EV3, and that shown in previous Fig. 2D, is the same. In order to display dynamic range in protein stability, we represented our data in log scale, so the reduced stability is rendered less apparent. In response to the referee's concern, we have revised our statement to *"Mutation to Gly* ***frequently*** *reduced the stability of those proteins* ***(18/36 proteins showed ≥15% reduction in stability)****, …."* **(p.10, ln.12-13)**.

Scales in Figure 3C, D and F are not readable and should be adjusted accordingly

We thank the referee for carefully proofreading our figures. We have adjusted the scales in all of our figures to ensure that they are readable.

Accepted **15th Dec 2020**

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Hsueh-Chi S Yen
Journal Submitted to: EMBO journal
Manuscript Number: 2020-105846

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We analyzed 20,000 or 100,000 individual cells for reporter cells carrying a single GPS construct or GPS peptide libraries, respectively. This sample size possesses sufficient statistical power to justify our findings.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In FACS experiments, we only measured red-positive cells (cells with GPS reporter) and excluded dead cells, cell debris, and multiple cells attached together.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	To minnimize bias caused by shRNA treatment-induced toxicity, each cell library separtely treated with shRNAs was analyzed by FACS according to three random samplings with 100,000 cells each. Data are presented as mean ± standard deviation (Fig. 1E and 1F).
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	We assessed the statistical significance and reported p-values of the findings as appropriates.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data in Fig 1B follows normal distributions according to Shapiro-Wilk normality tests (p-values > 0.05). The data in Fig 2B, 2C, 3A and Table EV2 have discrete counts following Poisson distributions.
Is there an estimate of variation within each group of data?	We calculated variance within each group of data in Fig 1B.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	We assessed the variances of data in Fig 1B and found that they were different between groups according to F-test. The Welch t-test was employed to adjust different group variances.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We describe the specific antibodies we used in our Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All of our cell lines were purchased from ATCC and tested for mycoplasma contamination. We describe the source of cell lines used in our Materials and Methods.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. 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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. 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.	NA
17. 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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	This study includes no data deposited in external repositories.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	This study includes no data deposited in external repositories.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA