THE
EMBO
JOURNAL

Collided ribosomes form a unique structural interface to induce Hel2-driven quality control pathways

Ken Ikeuchi, Petr Tesina, Yoshitaka Matsuo, Takato Sugiyama, Jingdong Cheng, Yasushi Saeki, Keiji Tanaka, Thomas Becker, Roland Beckmann and Toshifumi Inada.

Review timeline:

Submission date:	20th July 2018
Editorial Decision:	21st August 2018
Revision received:	19th November 2018
Accepted:	10th December 2018

Editor: Hartmut Vodermaier

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21st August 2018

Thank you again for submitting your manuscript on coupling of ribosome-associated quality control and no-go decay in di-ribosomes for our editorial consideration. Four referees with expertise in these respective topics have now evaluated it, with their reports copied below for your information. As you will see, all reviewers consider your findings interesting and potentially important. At the same time, they however list a number of specific issues that would need to be clarified prior to eventual publication. In particular, several experiments would require better description, rationalization, and/or quantification, and a major conceptual concern relates to the relative contributions of the two alternative pathways, which would require further investigation of physiological contexts in which the Not4-dependent effect mechanism actually occurs (see esp. referee 1 point 4 and referee 2 point 6). Furthermore, the referees also consider it important to better connect the structural and biochemical data within the manuscript. Finally, the reviewers ask for better discussion, overall presentation and more careful/factual interpretation (including alterations to the title!), as well as compliance with established nomenclature (main case: Hel2 instead of Rqt1).

Should you be able to satisfactorily address these key experimental and presentational issues, we would be happy to consider a revised version of this manuscript further for publication in The EMBO Journal.

REFEREE REPORTS.

Referee #1:

In the current manuscript under review, Ikeuchi and colleagues undertake a biochemical and structural study of the ribosome quality control pathway centered on the ubiquitin ligase, Hel2. The perform elegant structure function studies on Hel2 to map critical domains that are needed for either RNA cleavage (which they refer to as NGD) and Ltn1-mediated nascent chain ubiquitylation. They

are able to generate novel separation of function mutants of Hel2 that can complement the loss of Hel2 with regards to its NGD activity but not its ability to facilitate Ltn1-mediated RQC events. The authors nicely map mRNA cleavage events in cells lacking Hel2, or with Hel2 mutants, and in cells that lack critical lysine residues on uS10 that are normally ubiquitylated by Hel2. Interestingly, while mRNA cleavage events, and NGD in general, are completely abolished upon loss of Hel2, ubiquitin-site mutants in uS10 and loss of Rpt2/Slh1 results in alternative cleavage sites. The authors characterize this alternative pathway and show that Hel2, working in concert with Not4, can ubiquitylate eS7A/B to facilitate this alternative mRNA cleavage pattern. The authors also perform beautiful Cryo-EM structural characterization of a disome derived during translation of a stall-inducing sequence in vitro. The authors note several unique structural features of this disome and establish a model in which this unique disomic structure serves to initiate Hel2-mediated RQC and NGD events.
Overall, the data is this manuscript are beautiful and the authors claims are well-substantiated by the data. There are several new findings in this manuscript that will be of interest to the ribosome-quality control field as well as the larger protein homeostasis field. There are several minor alterations that would help establish the robustness of some of the authors' observations.
1- I would request that the authors use the original name, Hel2, for the ligase. Renaming it to Rqt1 only confuses the matter, especially with the human ortholog being named ZNF598. Indeed, the authors themselves are not consistent with using Rqt1 vs Hel2. I am not in favor of renaming genes to brand them.
2 - In the previous Nature Communication paper from Inada's group, they show that yeast that lack Hel2 are more sensitive to anisomycin by serial dilution growth assays. It would be very helpful to see which of their domain mutants rescue this growth phenotype. Do you need both the NGD and the RQC functions of Hel2 to restore this phenotype (i.e. does the 1-315 and the 1-438 versions for Hel2 both rescue? Or Neither? This assay would also be useful to examine the growth phenotype for the uS10 point mutant cells, as well as the cells lacking Slh1, or the newly identified eS7A mutant cells.
3- One of the more surprising results from this study is the demonstration that the point mutant uS10 cells (K6RK8R) show robust RNA cleavage compared to that observed in the Hel2 loss-of-function cells (Fig 2F,G,H). The authors to do some nice work showing that the cleavage pattern and cleavage sites are altered in this strain (and in the Slh1 delete strain) vs wild type. However, it is still surprising that the RNA is cleaved so robustly resulting in a substantial reduction in full length RNA, and that these cleavage events somehow are not productive and do not lead to down stream Ltn1-dependent ubiquitylation of the nascent chain. It would be useful to examine the actual RNA turnover using these mutant strains (similar to what was done in figure EV1D,F). Specifically, it would be critical to examine the turnover of the K(AAA)12 and R(CGN)12 reporters (full length) in the uS10 point mutant strain and in the Slh1 delete strain. Do these strains (which show robust RNA cleavage) still result in full NGD of the RNA. I would imagine so, but it would be worthwhile to measure this.
4 - The relevance of the alternative pathway utilizing Not4 and eS7A/B ubiquitylation is unclear. This pathway seems to only operate when uS10 is not ubiquitylated (but not when Hel2 is lost) and when Slh1 is lost. These conditions seem highly manipulated. Regardless, are there any observable phenotypes in the Not4 deletion strain or the eS7A point mutant strain? Are these strains sensitive to anisomycin? It looks like the cleavage of the 5' NGD-IM is altered in the Not4 deletion background? Is this relevant?

Referee #2:

In this manuscript, Ikeuchi and colleagues characterize the role of ribosomal protein ubiquitination by Rqt1 (hel2) on the process of no-go decay. Previous work by the same group showed Rqt1-mediated ubiquitination of ribosomal proteins to be critical for ribosome quality control (RQC) of nascent peptides as well as stalling. Here they expand on these observations by addressing the role of this process on mRNA surveillance, and in particular the endonucleolytic cleavage reaction that takes place during NGD. They find that cleavage on NGD reporters depends on Rqt1, which contradicts earlier reports by Saito et al. (Plos Genetics, 2015). They went on further to characterize the role of different domains of Rqt1 during RQC and NGD; by conducting truncation experiments

they were able to find a truncated protein that can separate the two processes. A mutant containing the zinc-finger domains but lacking the RING domain was functional for NGD but abolished RQC. The authors then went on to come up with a model that suggest there are two types of cleavage reactions: RQC+, which is dependent on uS10 ubiquitination, and RQC-, which is dependent on eS7 ubiquitination. The first one takes place just upstream of the leading ribosome, whereas the second one takes well further upstream. The authors then used cryoEM to solve the structure of a disome unit, which offered some clues about the interface between colliding ribosomes and how it may act as a platform for Rqt1 docking. Overall the manuscript offers some new and important insights into the mechanism of NGD and the role of ribosomal protein ubiquitination in triggering the endonucleolytic cleavage reaction. Having said that, there are some issues that need to be addressed for this paper to be suitable for publication. Most of these concerns have to do with the interpretation of the results and their presentation.

1) Most important is the title of the paper, which is misleading. While previous work form others suggested that collisions are important for RQC and NGD, there are no data in the current paper to support that the disome is the structural unit for RQC. As presented the paper provides structures of disomes, which give some potential hints for how Rqt1 may recognize stalled ribosomes at the interface between the collided ribosomes. Most of the in vitro ubiquitination assays were done with purified ribosomes that are not translating, so no collision can occur. I would suggest changing the title of the paper.

2) It is unclear why the authors used the ski2-E445Q to look at the effect of K63 polyubiquitination on NGD. The data presented in Figure 2A is not convincing. The cleavage products are not as evident as they are in the ski2 deletion.

3) The authors used Rqt1 overexpression to induce ubiquitination. Is this the only way to detect potential substrates? What about potential off-target effects.

4) It is clear that Rqt2 (slh1) deletion results in the accumulation of cleavage products of different sizes, but the equivalent sizes seen with the Rqt1 1-315 look similar to the wild-type ones. Also it is unclear why in the presence of Rqt1 1-439, NGD is completely inhibited (this truncation contains the Zinc Finger domains).

5) The effects reported on cleavage efficiencies need to be better quantified, especially when loading is an issue. The authors instead used qualitative analysis, which is sometimes not very obvious.

6) The claim of the two potential pathways for cleavage operating could be bolstered significantly by additional experiments. Mainly, their model would predict that deletion of not4 in the truncated rqt1 mutant background should completely abolish NGD.

7) The disomes for structural work were isolated without any apparent tricks; I may have missed it. How did the authors prevent cleavage from taking place? If no tricks were used, are they then looking at a population of ribosomes that avoided cleavage and hence are not representative of the real targets of Rqt1? Shouldn't have these been isolated in the absence of Rqt1?

Minor issues.

1) It is unclear why the half-lives of the NGD reporters in the rqt1Δ is much higher than that of the wild-type reporter.

Referee #3:

The ribosome is the final arbiter of gene expression, and recent years have seen a renaissance of interest in understanding the co-translational mechanisms governing protein and mRNA quality control. When ribosomes stall on a problematic mRNA, the messenger RNA is degraded in a process named NGD or No-Go Decay. Also, the nascent polypeptide may be degraded in a related but separable process known as RQC or Ribosome-associated Quality Control. In the RQC

response, arrested 80S ribosomes are split into large and small subunits, and this enables recognition and assembly of the ribosome-associated quality control complex (RQC), which targets nascent chains for CAT tailing, ubiquitination, and degradation. How the cell recognizes stalled ribosomes among the vast numbers of actively translating ribosomes remains poorly understood.

The present study concerns an apparent interplay between NGD and RQC. The authors report that the machinery controlling entry into the NGD and RGC pathways recognizes ribosome collisions. Specifically, the authors report that the "RQT" or RQC-Trigger complex-which includes the ubiquitin ligase Rqt1p/Hel2p (ZNF598 in mammals)-recognizes a pathological disome that comprises a leading but stalled 80S ribosome and the trailing ribosome that has collided with it. Functional and structural analysis of these disomes, using a series of reporter mRNAs that induce RQC or NGD, revealed fascinating structural details that explain published observations (for example, RQC/NGD dependence on Asc1/RACK1), and which may be responsible for recognition and thus downstream ubiquitination by the RQT on ribosome subunits uS10p and eS7p. In addition to an analysis of the ribosome-ribosome interactions related to stalling and turnover, the authors include descriptions of an alternative pathway that appears operative when RQT functions are disabled or deleted. This alternate pathway includes mRNA cleavage upstream of the leading disomes and is regulated by Not4p-mediated mono-ubiquitination of eS7p. Surprisingly, this upstream, alternate pathway does not apparently lead to RQC-mediated turnover of the nascent polypeptide, although that point may not be definitively established. Before final acceptance, I have a limited number of questions for the authors to consider addressing for the final version of their important study.

Major:
• I am sympathetic with the disappointment these authors must feel about the missing cryoEM density for Rqt1p. The manuscript may be easier to read if you just report the missing density directly and then address the remaining questions head-on. A simple statement like "we were unable to resolve density for Rqt1p for unclear reasons" would make it easier to discuss open questions and confusing aspects of the data. For example, perhaps Rqt1p recognizes still higher-order consequences of ribosome collisions, like a trisome or some other detail that a focus on the disomes-as interesting as the disome interaction is-missed. Perhaps the disome concept should be removed from the title, and replaced with ribosome collision as the key concept?
• Related to the above, without more mechanistic insight the mRNA cleavage mechanism, its dependence on ribosome splitting factors, and the remaining uncertainty about Rqct1p's structure and mechanism, I don't think we can readily explain the pattern of mRNA cleavages seen in Fig. 1E. For example, it appears that the P-site X1 cleavages still occur with reduced probability, even with the truncation Rqt1p over-expression rescue (Fig. 1E gel lane 9, X1 bands) I am especially intrigued and mystified by the 5' shift seen with the truncated form of Rqct1p. If there is a good explanation for the appearance of certain cut sites and the loss of others, please help me understand. If not, then a simple statement about future work to address these questions would help the reader not dwell too much on the details.
• I am also puzzled by the total protein levels in Fig. 1C versus D. If the mRNA is being cut by NGD factors, how can the cell make comparable amounts of total, full-length reporter protein? When I compare, for example, the 61-315 versus the 316-539 constructs for Rqt1p (lanes 21-24 in 1C), the total amount of read-through translation and thus full-length GFP-R(CGN)12-FLAG-HIS3 seems to be about the same, yet for one of these (Rqt1p61-315), the message is being cut (Fig. 1D, lane 11) and the other is not (Rqt1p316-539, Fig. 1D, lane 12). How is this possible? A brief acknowledgment and discussion of this puzzle seem warranted.
• What do the authors think happens to the nascent chains of all the ribosomes that are trailing the RQC-susceptible ribosomes? The idea that these nascent chains are NOT dealt with by CAT tailing and Ltn1p-mediated ubiquitination begs the question of how these ribosomes are cleared of their incomplete translation products.
• Rather than speculations about how Rqt1p and its associated E2's could serve as an E3 for both K48 and K63 linked chains, I would rather read about a well-understood precedent for an E3 ligase that is able to participate in multiple linkages patterns. I am not an expert in this area but perhaps
• I do not understand the mass spectrometry experiment in Figure EV10. The figure panels disagree with the figure legends and the methods: in some places, FLAG-GFP-stall-His3 appears to have been the bait, while in others to GFP-stall-FLAG-His3 appears to have been the bait. This obviously has huge implications for the expected results. Even if everything was done with the N'-terminal FLAG-GFP, I still don't understand how this experiment confirmed that only the leading ribosome

undergoes RQC. Please explain or remove.

Minor:
• Cite Brandman 2012 for the Asc1/RACK1 dependence of the RQC
• Some of the writing reads awkwardly in English. For example, "sites that are read by this disomes unit" doesn't make sense to me, I can't tell what the "reader" is. May I suggest simpler descriptions of the data, like "...the inclusion of residues 316-439 prevented this function"?
• Fig. EV2 suggests these were uS3 null cells? I didn't think that was possible since uS3/RPS3 is an essential gene. Please clarify?
• Seems a stretch to write: "Rqt1 seems to preferentially interact with colliding stalled ribosomes" since, unfortunately, the structural data does not reveal where or how Rqt1p binds ribosomes and a premise of the paper is that it binds disomes. I would avoid discussing Rqt1's interaction preferences until its binding mechanism is clarified.
• There is a citation to Fig. 5G, but no 5G that I could find, the relevant data seems to be 5F?
• I don't feel comfortable endorsing the vague reference to Rqt1p/Hel2p as an E4 activity. Even the notion of an E4 seems too poorly defined to be helpful here.
• 6H model figure: I recommend editing this so that it doesn't like there is a charged tRNA in the E-site. I would also consider showing uS10 ubiquitination (K48-linked) on the leading ribosome, and eS7 (K63-linked) on the following ribosome.
• There are several places where the degree or extent of poly-ubiquitination is compared, but there is ambiguity about when ubiquitination become poly-ubiquitination. For example, the authors wrote "Rqt1 was able to polyubiquitinate mono-ubiquitinated eS7A (Fig 5E, lane 5)" to describe an experiment that reveals a ladder with a max of 6 or 7 ubiquitin additions, similarly for Fig. 6F, where the ladder maxes out at ~5. By contrast, the authors interpreted Fig. 2E, lane 9, where the ladder maxes out at ~4 ubiquitin, appropriately as a failure of poly-ubiquitination in the absence of K63 availability. Perhaps the approximate threshold for poly-ubiquitin and its downstream consequences should be defined.

Referee #4:

In this manuscript the Inada group collaborating with the Beckmann group analyze the consequences of ribosome stalling. They investigate the impact of ribosome stalling on mRNA decay, fate of the synthesized proteins, ribosome modifications, and interaction between ribosomes using yeast as a model system. Their data provide evidence for different mechanisms leading to mRNA cleavage/decay, which is a new observation. Results presented in this manuscript provide new insights into the molecular roles of several factors implicated in these processes. In particular, the authors report a structural analyses of ribosome disomes where the first ribosome is paused and suggest how those may be involved in the ensuing RNA/protein decay processes.

The strong point of this manuscript is that it presents high quality data, in particular for the molecular mechanisms analyzed. The author's conclusions are thus well supported by the data presented. Some weaknesses are still present. In particular because some of the data sets presented are not tightly connected: hence, the relation of the disome structure presented with the molecular mechanisms detected remain speculative and is not tested experimentally. Validating the importance of the disome structure presented on NGD or RQC through specific experiments would have clearly improved/could improve the manuscript. Despite this limitation, I believe that the manuscript can be published in the EMBO Journal with some minor mandatory revisions.

Among those, the authors use non-canonical names for protein factors. For example, Rqt1 is used throughout the manuscript but not by others in the field. This name is not even referenced in the database of yeast standard names (SGD)! A situation in which each group uses its own nomenclature is only increasing confusion (e.g., searches on literature or general database will not retrieve all reports characterizing these factor). Authors should use standard gene names throughout their manuscript.
A second point that could be improved is the presentation of methods, pre-existing data and related work. Often references are indeed missing. For example:
- the method used to prepare the cell free translation extract that served to generate the structural data is not indicated (page 38, last line);

- evidence that ski2-E445Q is dominant negative is not indicated (page 10, 3 lines from bottom);
- molecular models docked in the EM envelope are not mentioned in the main text (page 13);
- sentences refereeing to previous work of the authors on Rtq1 bound ribosomes do not contain a reference (page 21, line 5), etc.

Careful reading the manuscript to add missing references is thus necessary before publication.
Authors should also provide more arguments on the validity of their experiments in the main text. Without going into details of the supplementary data and methods, it is difficult to understand by reading the manuscript whether the disome structure presented is representative of a large fraction of the structures observed by cryo EM and why it should be considered as informative. These arguments should be presented concisely in the manuscript, particularly in the absence of validating biological data. Indeed, it remains possible that this model represents only an artefactual structure formed in vitro.
Finally, the discussion could probably be reinforced e.g., through indications why and how the CCR4-NOT complex could be involved in this process.

Point-by-point Response

First of all, we thank the editor and referees for your constructive comments and input. We have addressed all your comments by our point-by-point responses indicated below in "Response to Referee" sections. The revised manuscript also contains minor modifications throughout to correct typos and increase clarity in some places.

Response to Referee #1:

Overall, the data in this manuscript are beautiful and the authors claims are well-substantiated by the data. There are several new findings in this manuscript that will be of interest to the ribosome-quality control field as well as the larger protein homeostasis field. There are several minor alterations that would help establish the robustness of some of the authors' observations.

1 - I would request that the authors use the original name, Hel2, for the ligase. Renaming it to Rqt1 only confuses the matter, especially with the human ortholog being named ZNF598. Indeed, the authors themselves are not consistent with using Rqt1 vs Hel2. I am not in favor of renaming genes to brand them.

> We agree and consistently use Hel2 instead of Rqt1 now in order to avoid confusion. However, we mention the "Rqt1" denomination in the introduction section as "Hel2/Rqt1" once, since we introduced "Rqt" names in our previous study to describe the functional "RQT-complex" composed of Slh1(Rqt2), Cue3(Rqt3) and Ykr023w (Rqt4) (Matsuo et al., Nat. commun., 2017). We registered the RQT4 as a standard name of YKR023W in SGD.

2 - In the previous Nature Communication paper from Inada's group, they show that yeast that lack Hel2 are more sensitive to anisomycin by serial dilution growth assays. It would be very helpful to see which of their domain mutants rescue this growth phenotype. Do you need both the NGD and the RQC functions of Hel2 to restore this phenotype (i.e. does the 1-315 and the 1-438 versions for Hel2 both rescue? Or Neither? This assay would also be useful to examine the growth phenotype for the uS10 point mutant cells, as well as the cells lacking Slh1, or the newly identified eS7A mutant cells.

> We thank the referee for this constructive suggestion: To explore the growth phenotypes, we performed spot assays for the Hel2 domain mutants to assess anisomycin sensitivity, and our results are shown in the new Appendix Figure S1. Indeed, Hel2 1-539 and 61-539 as well as full-length Hel2, which are sufficient for RQC induction, rescued the anisomycin sensitivity of *hel2* deletion cells. On the other hand, as you mentioned above, 1-439, 1-315, 61-439 and 61-315 mutants of Hel2, which are not sufficient for RQC induction, could not rescue the anisomycin sensitivity of *hel2* deletion cells. Together, the anisomycin sensitivity of Hel2 mutants are correlated perfectly with their ability to induce RQC, but not with their NGD^{RQC-} induction abilities.
> To address these new insights on anisomycin sensitivity in the manuscript, we added a paragraph in the results section (page 10, line 4-11):
> "We have previously described an interesting phenotype of sensitivity to the anisomycin translation elongation inhibitor in RQC-deficient cells (Matsuo et al., 2017). Intriguingly, Hel2 mutants that were unable to trigger RQC (1-315, 61-315, 1-439 and 61-439) remained sensitive to anisomycin, whereas RQC-competent Hel2 mutants (1-539, 61-539) were not susceptible to this drug (Appendix Fig. S1). Anisomycin binds in the ribosomal A-site and most likely prevents tRNA accommodation, which leads to stalling and ribosome collisions. Thus, it could trap ribosomes stalled in a state which occurs during

RQC (presumably a disome) and increase RQC turnover demand."
The referee may have overlooked that we have already performed spot assays of *slh1*Δ and *uS10-K6/8R* mutant cells in the presence of anisomycin described in Matsuo *et al*., Nature Communications, 2017. We found that *slh1*Δ and *uS10-K6/8R* mutants were sensitive to anisomycin, again correlating with RQC inducing capability. To make this information readily available to readers, we have added the previously cited sentence to the revised manuscript:
"We have previously described an interesting phenotype of sensitivity to the anisomycin translation elongation inhibitor in RQC-deficient cells (Matsuo et al., 2017)."

In addition, in the revised version of our manuscript we provide new experiments showing that both the *not4*Δ and *eS7A-4KR* mutant do not exhibit anisomycin sensitivity (Figure EV4A). These results are consistent with the Hel2 domain analyses demonstrating a correlation of anisomycin susceptibility with the ability to induce RQC and NGD^{RQC+}.

3- One of the more surprising results from this study is the demonstration that the point mutant uS10 cells (K6RK8R) show robust RNA cleavage compared to that observed in the Hel2 loss-of-function cells (Fig 2F, G, H). The authors to do some nice work showing that the cleavage pattern and cleavage sites are altered in this strain (and in the Slh1 delete strain) vs wild type. However, it is still surprising that the RNA is cleaved so robustly resulting in a substantial reduction in full length RNA, and that these cleavage events somehow are not productive and do not lead to down-stream Ltn1-dependent ubiquitylation of the nascent chain. It would be useful to examine the actual RNA turnover using these mutant strains (similar to what was done in figure EV1D, F). Specifically, it would be critical to examine the turnover of the K(AAA)12 and R(CGN)12 reporters (full length) in the uS10 point mutant strain and in the Slh1 delete strain. Do these strains (which show robust RNA cleavage) still result in full NGD of the RNA. I would imagine so, but it would be worthwhile to measure this.

We thank the referee for this suggestion. As requested, we quantified the level of full-length reporter mRNA and 5'-NGD intermediates as now shown in the revised Figure 7A and 7B. The 5'-NGD intermediates were indeed increased in *slh1*Δ cells (1.7-fold) and *uS10-K6/8R* mutant cells (2.3-fold), and the level of full-length mRNA was slightly decreased in both cell lines compared to wild type. We also measured half-lives of full-length reporter mRNA as now shown in Figure EV1E. The half-lives of reporter mRNAs containing R(CGN)12 or K(AAA)12 arrest-inducing sequences are not prolonged in *slh1*Δ and *uS10-K6/8R* mutant cells compared to wild-type cells. These results indicate that the full-length reporter mRNAs with arrest inducing sequences are still degraded with normal decay rate (are subject to full NGD), even in the absence of Slh1 and the ubiquitination of uS10, as mentioned by the referee.

4 - The relevance of the alternative pathway utilizing Not4 and eS7A/B ubiquitylation is unclear. This pathway seems to only operate when uS10 is not ubiquitylated (but not when Hel2 is lost) and when Slh1 is lost. These conditions seem highly manipulated.

We thank the referee for this comment. Under the described conditions, the Not4 and eS7-dependent pathway of NGD^{RQC-} is indeed highly activated. However, it is also active, albeit at a lower level, in the presence of Slh1 and ubiquitination of uS10 as confirmed by the presence of the upstream cleavage sites (blue arrowhead in Figure 1E lane 6 and Figure 2I lane 11), that are detectable even under wild type conditions in a *xrn1*Δ background (as designated "wild-type" for primer extension experiments).

Moreover, as mentioned in the answer to your comment #3, the mRNA half-life of K(AAA)12 and R(CGN)12 reporters were not affected under the uS10 point mutant and *slh1* deletion conditions. This showed that NGD could be as efficient in the alternative NGD^{RQC-} pathway as in the NGD^{RQC+} pathway, which also argues in favour of physiological relevance. Therefore, the alternative NGD^{RQC-} pathway may not only provide redundancy in a critical physiological process but, in addition, may constitutively complement canonical NGD.

To clarify this to the readers, we have added the following text passage to the discussion in the revised manuscript (page 23, line 12):

"While this upstream-acting NGD^{RQC-} would easily be considered as "alternative" or "non-canonical" pathway active only under RQC-deficient conditions, it is important to notice that its products are readily detectable in a wild-type background (*xrn1*Δ) as seen in Fig 1E lane 6 and Fig 2I lane 11. Moreover, as demonstrated in Fig EV1E, the mRNA half-lives of $K(AAA)_{12}$ and $R(CGN)_{12}$ reporters were not affected under the RQC-deficient uS10 point mutant and Slh1 deletion conditions. This showed that NGD could be as efficient in the alternative NGD^{RQC-} pathway as in the "canonical" NGD^{RQC+} pathway, which strongly argues in favour of its physiological relevance. We therefore propose, that the NGD^{RQC-} pathway may not only provide redundancy in a critical physiological process of NGD but also constitutively contribute to its overall efficiency. This is further supported by results presented in Fig EV4A, where we showed genetic interaction between Not4 and Hel2 or Slh1 and the necessity of at least one working NGD pathway for cell viability"

Regardless, are there any observable phenotypes in the Not4 deletion strain or the eS7A point mutant strain? Are these strains sensitive to anisomycin?

We followed the referee's request and checked Not4 and eS7A-4KR mutant cells for growth and anisomycin sensitivity. Indeed Not4 and eS7A-4KR mutant cells exhibited a severe growth defect under normal culture conditions (YPD media, 30ºC; Figure EV4A). In addition, *not4*Δ*hel2*Δ or *not4*Δ*slh1*Δ double deletion strains exhibited synthetic growth defects (Figure EV4B). These results indicate that there is a genetic interaction between Not4 and Hel2 or Slh1, and that one of the NGD pathways would be required for cell viability. Additionally, *not4*Δ cells and *eS7A-4KR* mutant cells are not sensitive to anisomycin, suggesting that Not4 and ubiquitination of eS7A are not required for RQC like Slh1 and ubiquitination of uS10.

The following text passage was added to the revised manuscript Result section to describe the obtained results (page 21, line 14):

"To assess the physiological relevance of the two NGD pathways, RQC-coupled NGD (NGD^{RQC+}) and Not4-dependent NGD (NGD^{RQC-}) taking place upstream of the disome, we examined the growth and the anisomycin sensitivity of *not4*Δ and *eS7A-4KR* mutant cells. As shown above, anisomycin sensitivity can be used as a readout to distinguish NGD^{RQC+} from NGD^{RQC-}. Deletion of Not4 and eS7A-4KR mutation caused a significant growth defect compared to the wild-type cells (Fig EV4A). Despite the severe growth defect, anisomycin did not further affect the growth of *not4*Δ and *eS7A-4KR* mutant cells (Fig EV4A), indicating that Not4 and ubiquitination of eS7A are not directly associated with RQC. Intriguingly, knocking out both NGD^{RQC+} and NGD^{RQC-} using a *not4*Δ*hel2*Δ or a *not4*Δ*slh1*Δ double mutation displayed a synthetic growth defect (Fig EV4B). This further indicates that Not4 may be involved in the Hel2-independent mRNA quality control pathway and play a crucial role in the absence of Hel2-mediated quality controls. We also found that *not4*Δ*xrn1*Δ double deletion mutations conferred synthetic lethality (Fig EV4C), suggesting that Hel2-independent mRNA quality control pathway

might be largely required for cell growth when Xrn1-mediated decay is defective."
These results are now also discussed in Discussion as cited in the previous answer.

It looks like the cleavage of the 5' NGD-IM is altered in the Not4 deletion background? Is this relevant?

The referee is right that the major 5'-NGD intermediate is longer in Not4 deletion cells (Figure 7A), which is in agreement with our finding that Not4 is important for cleavages upstream of the stalled di-ribosome, in particular in the NGD^{RQC-} pathway. The observation of a longer 5'NGD-IM is therefore relevant by confirming the Not4 dependency of NGD^{RQC-}.

(Unfortunately, the determination of the exact cleavage sites of 5'-NGD-IM in *xrn1Δnot4Δ* cells was impossible, however, since *xrn1Δnot4Δ* double mutations exhibited synthetic lethality (SL) probably due to a combined impact on canonical mRNA turnover (Figure EV4C).)

To make this important detail clear for the readers we have modified the legend of Fig 7A to highlight this information (page 33, line 15):
"Note the size difference of 5'-NGD IMs resulting from NGD^{RQC+} and NGD^{RQC-} and that the shorter 5'-NGD intermediate (representing intermediates of the NGD^{RQC-} pathway) was reduced in *not4Δslh1Δski2Δ* mutant cells."

Response to Referee #2:

Overall the manuscript offers some new and important insights into the mechanism of NGD and the role of ribosomal protein ubiquitination in triggering the endonucleolytic cleavage reaction. Having said that, there are some issues that need to be addressed for this paper to be suitable for publication. Most of these concerns have to do with the interpretation of the results and their presentation.

1) Most important is the title of the paper, which is misleading. While previous work form others suggested that collisions are important for RQC and NGD, there are no data in the current paper to support that the disome is the structural unit for RQC. As presented the paper provides structures of disomes, which give some potential hints for how Rqt1 may recognize stalled ribosomes at the interface between the collided ribosomes. Most of the in vitro ubiquitination assays were done with purified ribosomes that are not translating, so no collision can occur. I would suggest changing the title of the paper.

> We agree with the referee that the title of the paper might have been misleading when strictly referring to disomes, especially when considering only the initially presented data. As also pointed out by referee #3, higher order oligoribosomes could play a role as signalling hub for downstream quality control pathways. However, our new data as well as a recent joint publication by the Hegde and Ramakrishnan groups (Juszkiewicz et al., 2018) suggest that formation of a disome with a defined structure indeed serves as a minimal unit for molecular recognition by Hel2 and subsequent induction of quality control pathways.
> In our revised manuscript we added an experiment directly proving that stalled disomes are more efficiently ubiquitinated than monosomes by Hel2 (Fig. 3E). Nevertheless, since this experiment doesn't exclude the possibility that higher order oligoribosomes are also recognized by Hel2 as pointed out by referee #3, we followed the suggestion of both referees and changed the title accordingly to:
>
> "Collided ribosomes form a unique structural interface to induce Hel2-driven quality control pathway"

2) It is unclear why the authors used the ski2-E445Q to look at the effect of K63 polyubiquitination on NGD. The data presented in Figure 2A is not convincing. The cleavage products are not as evident as they are in the ski2 deletion.

> The ski2-E445Q mutant was used for its dominant-negative effect on activity of Ski2 as described in our previous study (Ikeuchi and Inada, Sci. Rep., 2016). We also used the dominant-negative Ski2-E445Q mutant in Appendix Figure S8 to detect 5'NGD intermediate in *not4Δhel2Δ* double mutant background while avoiding synthetic lethality (as discussed below).
> In order to improve the clarity of results in Figure 2A, we constructed *ski2ΔUb-WT* and *ski2Δub-K63R* strains and replaced the previously obtained data (Figure 2A of the revised version).
> We have also included the explanation and reference to our previous work in the revised manuscript to clarify the use of Ski2-E445Q (page 21, line 5):
> "Additionally, we confirmed that Not4-dependent mRNA cleavages in NGD^{RQC-} were not restored by expressing the Hel2(1-315) mutant (Appendix Fig S8D). However, this phenotype could only be confirmed by using the overexpression of Ski2-E445Q as a dominant negative Ski2 mutant (Ikeuchi & Inada, 2016; Appendix Fig S8) instead of *ski2* deletion background. This was caused by synthetic lethality induced in the *ski2Δhel2Δnot4Δ* strain."

3) *The authors used Rqt1 overexpression to induce ubiquitination. Is this the only way to detect potential substrates? What about potential off-target effects.*

We agree with the referee in sharing the concern of off-target effects. Yet, overexpression of Hel2 was actually not the only way to detect substrates and was only used for initial screening (Figure 6A and Appendix Figure S7). As most clearly visible for uS10 and uS3 in Figure 6A the same ubiquitinated bands are detectable even before overexpression of Hel2. In order to further verify these proteins as Hel2 substrates, we checked whether the ubiquitination of the identified targets disappeared in a *hel2*Δ background. Moreover, we also generated K-R substitution mutants of target residues to check the RQC/NGD phenotype using our reporter systems, which is all done under wild type expression levels of Hel2 (e. g. Figure 2C, 6C and 6D). As an additional line of evidence we studied the role of Hel2 in uS10 and eS7A ubiquitination in an *in vitro* ubiquitination reaction as shown in Figures 2E, 6E, 6F and 7G. Taken together, all results indicate strongly that uS10 and eS7 are substrates for Hel2, and are not generated by off-target effects.

4) It is clear that Rqt2 (slh1) deletion results in the accumulation of cleavage products of different sizes, but the equivalent sizes seen with the Rqt1 1-315 look similar to the wild-type ones. Also it is unclear why in the presence of Rqt1 1-439, NGD is completely inhibited (this truncation contains the Zinc Finger domains).

We thank the referee for pointing out this seeming inconsistency. This confusion was caused by using different parameters when performing electrophoresis. In Figure 1D, we performed electrophoresis at 200 V for 40 minutes using a 1.2% agarose gel, while in Figure 2 and later, we performed electrophoresis at 200 V for 50 minutes using a 2% agarose gel. This is the reason why the cleavage products seen for Hel2(1-315) seem to be similar to the wild-type ones. In the revised version, we repeated the experiments using the uniform electrophoresis conditions (Figure EV2). Here, the expected uniform presence of slightly shorter 5'NGD-IM and longer 3'NGD-IM is revealed for both, the Hel2(1-315) and the slh1 deletion. Moreover, we performed primer extension experiments to determine the nature of cleavages in Hel2(1-315) expressing cells (Figure 1E) confirming a more upstream cleavage site explaining shorter 5'NGD-IM and corresponding longer 3'NGD-IM.
Hel2(1-439) indeed could not induce NGD while the 1-315 version of Hel2 could. We do not understand the phenotype of Hel2(1-439) mutant cells, however, one possibility is that the 316-439 region acts as auto-inhibitor in the Hel2(1-439) mutant. In general, it is not unusual that certain mutations result in inactivation of proteins due to autoinhibition or misfolding.
This issue is discussed in the Discussion section (page 26, line 4):
"Deletion analysis suggests that residues 315-439 of Hel2 inhibit the activity of Hel2(1-315) in the polyubiquitination of eS7. We suspect that the interaction of this putative auto-inhibitory domain with the stalled polysome allows the zinc finger domains to bind to an acceptor ubiquitin."

5) The effects reported on cleavage efficiencies need to be better quantified, especially when loading is an issue. The authors instead used qualitative analysis, which is sometimes not very obvious.

We appreciate this relevant request which was also pointed out by referee #1. As we discussed above (comment #3 from referee #1), we performed and added quantitative analysis of northern blotting results in the revised Figure 7, in which the levels of *SCR1* should be clearly distinguishable. We hope that

this will further substantiate the reported effects on cleavage efficiencies as requested.

6) The claim of the two potential pathways for cleavage operating could be bolstered significantly by additional experiments. Mainly, their model would predict that deletion of not4 in the truncated rqt1 mutant background should completely abolish NGD.

This is a very pertinent suggestion. To accommodate this request, we tried to construct a *ski2*Δ*not4*Δ*hel2*Δ triple deletion strain to assess the function of Hel2(1-315) mutant in *not4*Δ background. However, this was not possible due to synthetic lethality. Therefore, instead of the triple deletion strain, we used *not4*Δ*hel2*Δ cells expressing the Ski2-E445Q dominant negative mutant. Using this strain, we found that the deletion of Not4 in the truncated *hel2/rqt1* mutant background completely abolished NGD as shown in Appendix Figure S8.

7) The disomes for structural work were isolated without any apparent tricks; I may have missed it. How did the authors prevent cleavage from taking place? If no tricks were used, are they then looking at a population of ribosomes that avoided cleavage and hence are not representative of the real targets of Rqt1? Shouldn't have these been isolated in the absence of Rqt1?

We thank the referee for this question. Indeed, no "tricks" were used to produce the disome sample for structural analysis. We used our self-made *in vitro* translation system and extracts were generated from a strain, where Ski2, a component of the 3'-5' mRNA degradation machinery is deleted. This leads to stabilization of mRNAs to be translated in our extract. In our experimental setup, we add this mRNA in a relatively high excess, which most likely overloads the quality control systems. This allowed us to isolate the stalled NGD/RQC substrate despite having all the endogenous response-mediating proteins present in the mixture. To increase the clarity of sample preparation procedure, we have modified the whole "Preparation of the CGA-CCG-stalled mono- and disomes" Methods section (page 43, line 11) and included following description:
"*In vitro* translation was performed at 17 °C for 75 min using great excess of template mRNA (38 µg per 415 µl of extract) to prevent degradation of resulting stalled ribosomes by endogenous response factors."

The referee is right to question if these disomes are the real substrate for Hel2. To address this better, we performed an additional in vitro ubiquitination experiment (as already mentioned in answer to comment #1) which confirms the disomes used for the structural study as targets of Hel2 (Figure 3E). A structure of Hel2 bound to colliding ribosomes is the next major step and obtaining di- or oligoribosomes from Hel2 deletion strain might be a key to solve such a structure. However, this is beyond the scope of this work.

Minor issues.

1) It is unclear why the half-lives of the NGD reporters in the rqt1Δ is much higher than that of the wild-type reporter.

This effect is caused by the fact that the GFP-FLAG-HIS3 (no insertion) reporter system does not induce the No-Go Decay machinery. On the other hand, R(CGN)12 or K(AAA)12 containing reporters are mainly degraded by NGD machinery. Under NGD-defective conditions, the R(CGN)12 or K(AAA)12 containing reporters are stabilized but the GFP-FLAG-HIS3 (no

insertion) reporter remains efficiently degraded by the general mRNA turnover machinery (Figure EV1E).

Response to Referee #3:

Major:

• I am sympathetic with the disappointment these authors must feel about the missing cryoEM density for Rqt1p. The manuscript may be easier to read if you just report the missing density directly and then address the remaining questions head-on. A simple statement like "we were unable to resolve density for Rqt1p for unclear reasons" would make it easier to discuss open questions and confusing aspects of the data.

In contrast to the referee's suspicion we actually aimed for accumulating disomes and had little hope to catch Hel2 assuming a rather transient interaction. Nevertheless, observing Hel2 on the disome would have been ideal and we agree to address this issue as requested.
Following text passage is now included in the main text (page 25, line 6):
"Since we did not detect any Hel2 density in our sample, we cannot rule out the possibility that it stably recognizes a higher order stalling interface (such as trisome or tetrasome). Nonetheless, during the peer review process of our manuscript, a study by the Hegde and Ramakrishnan groups (Juszkiewicz et al., 2018) was published, confirming that formation of a collided rabbit disome unit with a defined structure indeed serves as a platform for molecular recognition by the mammalian homologue of Hel2."

For example, perhaps Rqt1p recognizes still higher-order consequences of ribosome collisions, like a trisome or some other detail that a focus on the disomes-as interesting as the disome interaction is-missed. Perhaps the disome concept should be removed from the title, and replaced with ribosome collision as the key concept?

We agree with the referee that higher order oligoribosomes may represent an even better Hel2 substrate. This aspect was reflected in the Discussion and is cited in the previous answer. Our previous data indeed show a distribution of Hel2 in the polyribosome region of the gradient with enrichment on polysomes (Matsuo et al., 2017). As already mentioned before, we performed an *in vitro* assay showing that Hel2 preferentially ubiquitinates uS10 on a stalled disome (Figure 3E) rather than monosome. Nevertheless, we followed the referee's request and modified the title to highlight the key collision concept as follows:

"Collided ribosomes form a unique structural interface to induce Hel2-driven quality control pathway"

• Related to the above, without more mechanistic insight the mRNA cleavage mechanism, its dependence on ribosome splitting factors, and the remaining uncertainty about Rqet1p's structure and mechanism, I don't think we can readily explain the pattern of mRNA cleavages seen in Fig. 1E. For example, it appears that the P-site X1 cleavages still occur with reduced probability, even with the truncation Rqt1p over-expression rescue (Fig. 1E gel lane 9, X1 bands). I am especially intrigued and mystified by the 5' shift seen with the truncated form of Rqt1p. If there is a good explanation for the appearance of certain cut sites and the loss of others, please help me understand. If not, then a simple statement about future work to address these questions would help the reader not dwell too much on the details.

We totally agree that we do not fully understand these events mechanistically. Under normal conditions the favoured cutting sites X1 to X4 are protected by the disome unit and we do not know how exactly these cleavages are carried

out. We agree with the referee and mention that in the text accordingly, that future research has to address that question.
The mysterious 5' shift (upstream of the proposed colliding ribosome) induced by the Hel2 truncation or by the *slh1* deletion is also not understood with respect to which nuclease activity is responsible and why the canonical X1 to X4 cleavages are suppressed. However, here we can show that due to truncated Hel2 or *slh1* deletion, canonical RQC and concomitant splitting of the stalled ribosome is inhibited and instead, the upstream cutting sites are preferentially employed. Again, the exact mechanism of this event will be the subject of future research, in particular, the identification of the enigmatic 5' endonuclease activity. We inserted these paragraphs into the main text Discussion, as requested (page 23, line 1):
"Through our mutational analysis of Hel2 we could dissect the NGD pathway into two branches. The first branch was coupled to RQC (NGD^{RQC+}) and lead to cleavage events in the mRNA covered by the first two stalled ribosomes (disome unit) The second branch was uncoupled from RQC (NGD^{RQC-}) and resulted in upstream cleavage events on the mRNA outside of the disome unit and potentially covered by following ribosomes. The exact mechanisms of these cleavage events will be the subject of future research, in particular regarding the identification of the long enigmatic endonuclease responsible for this observed activity."

• I am also puzzled by the total protein levels in Fig. 1C versus D. If the mRNA is being cut by NGD factors, how can the cell make comparable amounts of total, full-length reporter protein? When I compare, for example, the 61-315 versus the 316-539 constructs for Rqt1p (lanes 21-24 in 1C), the total amount of read-through translation and thus full-length GFP-R(CGN)12-FLAG-HIS3 seems to be about the same, yet for one of these (Rqt1p61-315), the message is being cut (Fig. 1D, lane 11) and the other is not (Rqt1p316-539, Fig. 1D, lane 12). How is this possible? A brief acknowledgment and discussion of this puzzle seem warranted.

Although we used the same stall-inducing $R(CGN)_{12}$ sequence, the seeming contradiction is produced by the fact that the protein levels detected in Figure 1C are not directly associated with the levels of full-length mRNA and its cleavage efficiency. Full-length protein production is dependent on the equilibrium between levels of full-length mRNA, translational stalling efficiency and RQC induction efficiency. Therefore, full-length levels alone do not necessarily correlate with full-length protein levels.
Moreover, the read-out here is semi-quantitative and used by us for determining the principle presence of RQC or NGD activity under the given conditions.

• What do the authors think happens to the nascent chains of all the ribosomes that are trailing the RQC-susceptible ribosomes? The idea that these nascent chains are NOT dealt with by CAT tailing and Ltn1p-mediated ubiquitination begs the question of how these ribosomes are cleared of their incomplete translation products.

We no longer claim that exclusively the leading ribosome undergoes RQC (as based on the deleted mass spectrometry results). It is clear, that the nascent chains in the trailing ribosomes cannot be released by canonical termination due to a lack of a stop codon. Therefore, the authors think that the trailing ribosomes will have to be split one or the other way (either involving Hbs1-Dom34-ABCE1 or the RQT system) leaving behind a 60S subunit carrying a peptidyl-tRNA. We further think that this will be recognized as substrate and thereby dealt with by the RQC factors.

• Rather than speculations about how Rqt1p and its associated E2's could serve as an E3 for both K48 and K63 linked chains, I would rather read about a well-understood precedent for an E3 ligase that is able to participate in multiple linkages patterns. I am not an expert in this area but perhaps.

As suggested, we added to the Discussion an example of a well-understood precedent of an E3 ligase that is able to participate in multiple linkages patterns. In addition, we would like to point out that we previously reported that the polyubiquitination of uS10 is K48-linked (Matsuo et al., 2017), and clearly demonstrate (and do not speculate) in this study, that the K63-linked poly-ubiquitination by Hel2 is required for NGD and RQC. Therefore, Hel2 acting as an E3 for both K48 and K63 linked chains is not unprecedented and clearly concluded from our data.
The discussion section was modified starting page 26, line 12 to include following description:
"We previously reported that the polyubiquitination of uS10 is K48-linked (Matsuo et al., 2017). In this study we demonstrated the K63-linked poly-ubiquitination by Hel2, indicating that Hel2 forms both K48-linked and K63-linked poly ubiquitin chain on uS10 at K6 or K8 residues. Other E3 ligases have also been described to connect ubiquitin chains via multiple linkage types. For instance, a well-established E3 ligase Parkin is able to connect ubiquitin chains through K11, K48 and K63 (reviewed in Yau & Rape, 2016)."

• I do not understand the mass spectrometry experiment in Figure EV10. The figure panels disagree with the figure legends and the methods: in some places, FLAG-GFP-stall-His3 appears to have been the bait, while in others to GFP-stall-FLAG-His3 appears to have been the bait. This obviously has huge implications for the expected results. Even if everything was done with the N'-terminal FLAG-GFP, I still don't understand how this experiment confirmed that only the leading ribosome undergoes RQC. Please explain or remove.

In general, it is difficult to determine a peptide containing methionine residue with a small number of charged residues by LC-MS/MS, such as peptide derived from colliding ribosome in our case. As you suggested, we decided to remove this figure (previous Figure EV10) in the revised version.

Minor:
• Cite Brandman 2012 for the Asc1/RACK1 dependence of the RQC.

As requested, we inserted the reference in the following (page 24, line 13).

• Some of the writing reads awkwardly in English. For example, "sites that are read by this disomes unit" doesn't make sense to me, I can't tell what the "reader" is. May I suggest simpler descriptions of the data, like "...the inclusion of residues 316-439 prevented this function"?

According to your suggestion, we modified the sentences as follows:
"We show that endonucleolytic cleavage of NGD reporter mRNA occurs at sites within this disome unit."
"Moreover, this singular function in NGD seemed to be inhibited by the Hel2 region comprising residues 316-439."
Our revised version was thoroughly edited by a biochemist, who is a native speaker of English as now stated in Acknowledgements.

• Fig. EV2 suggests these were uS3 null cells? I didn't think that was possible since uS3/RPS3 is an essential gene. Please clarify?

As you mentioned, uS3 is an essential gene. In Appendix Figure S2 in revised version, we utilized *ski2*Δ*uS3*Δ strain expressing uS3 wild-type or mutant (e.g. K212R) from plasmid, which means the genomic *SKI2* and *uS3* were disrupted but the strain was always rescued by plasmid-derived *uS3*.
To improve clarity for readers, we have modified the figure legend to specifically include this information:
"The *GFP-R(CGN)*$_{12}$*-HIS3* mRNA (FL) and 5' NGD-intermediate (5'NGD-IM) were detected by Northern blot analysis in the *ski2*Δ*uS3*Δ strains expressing the indicated plasmid-derived uS3 mutant proteins with the DIG-labelled *GFP* probe."

• Seems a stretch to write: "Rqt1 seems to preferentially interact with colliding stalled ribosomes" since, unfortunately, the structural data does not reveal where or how Rqt1p binds ribosomes and a premise of the paper is that it binds disomes. I would avoid discussing Rqt1's interaction preferences until its binding mechanism is clarified.

According to the referee's suggestion, we modified this text passage at page 24, line 20 to reduce its speculative character and only point out our previous results:
"However, it is unclear which of the ribosomes is poly-ubiquitinated and where exactly Hel2 binds. Our previous study showed that affinity-purified Hel2-ribosome complexes mainly yielded 80S ribosomes in the rotated state (Matsuo et al., 2017), suggesting that Hel2 may preferentially bind colliding rotated ribosomes as a primary contact site (Fig EV5)."

• There is a citation to Fig. 5G, but no 5G that I could find, the relevant data seems to be 5F?

Thank you for pointing this out. We have corrected the mistake.

• I don't feel comfortable endorsing the vague reference to Rqt1p/Hel2p as an E4 activity. Even the notion of an E4 seems too poorly defined to be helpful here.

We agree that Hel2 description as an E4 enzyme could be confusing for readers. In the revised version, we omitted this notion in accordance with your suggestion.

• 6H model figure: I recommend editing this so that it doesn't like there is a charged tRNA in the E-site.

Thank you for pointing this out. We have corrected the mistake.

I would also consider showing uS10 ubiquitination (K48-linked) on the leading ribosome, and eS7 (K63-linked) on the following ribosome.

According to your suggestion, we modified the Figure 7H in the revised version and show uS10 ubiquitination on the leading ribosome in a disome, and eS7 on the colliding ribosome upstream of a disome. Note that, in this study, we indeed showed that K63-linked uS10 ubiquitination is required for RQC and NGD^{RQC+}, but we do not have any evidence that K48-linked uS10 ubiquitination is required for or functional in RQC and NGD^{RQC+}. The function of K48-linked uS10 ubiquitination should be uncovered by further experiments in future.

• There are several places where the degree or extent of poly-ubiquitination is compared, but there is ambiguity about when ubiquitination become poly-ubiquitination. For example, the authors wrote "Rqt1 was able to polyubiquitinate mono-ubiquitinated eS7A (Fig 5E, lane 5)" to describe an experiment that reveals a ladder with a max of 6 or 7 ubiquitin additions, similarly for Fig. 6F, where the ladder maxes out at ~5. By contrast, the authors interpreted Fig. 2E, lane 9, where the ladder maxes out at ~4 ubiquitin, appropriately as a failure of poly-ubiquitination in the absence of K63 availability. Perhaps the approximate threshold for poly-ubiquitin and its downstream consequences should be defined.

As commonly done in the field, we distinguish between three categories, namely mono-, di- and poly-ubiquitination, with the definition of polyubiquitination being more than two ubiquitin units on the substrate.
Indeed, the number of ubiquitin units in the poly-ubiquitin chains varies in the different experiments. It is generally difficult to properly detect heavier poly-ubiquitin chains in *in vivo* samples, since poly-ubiquitin chains are subjected to quick deubiquitinase activity. On the other hand, poly-ubiquitin chains were easily detected in *in vitro* reaction samples, because of low contamination by deubiquitinases. We modified the figures and text for clearer description according to your suggestion.

Response to Referee #4:

In this manuscript the Inada group collaborating with the Beckmann group analyze the consequences of ribosome stalling. They investigate the impact of ribosome stalling on mRNA decay, fate of the synthesized proteins, ribosome modifications, and interaction between ribosomes using yeast as a model system. Their data provide evidence for different mechanisms leading to mRNA cleavage/decay, which is a new observation. Results presented in this manuscript provide new insights into the molecular roles of several factors implicated in these processes. In particular, the authors report a structural analyses of ribosome disomes where the first ribosome is paused and suggest how those may be involved in the ensuing RNA/protein decay processes.

The strong point of this manuscript is that it presents high quality data, in particular for the molecular mechanisms analyzed. The author's conclusions are thus well supported by the data presented. Some weaknesses are still present. In particular because some of the data sets presented are not tightly connected: hence, the relation of the disome structure presented with the molecular mechanisms detected remain speculative and is not tested experimentally. Validating the importance of the disome structure presented on NGD or RQC through specific experiments would have clearly improved/could improve the manuscript. Despite this limitation, I believe that the manuscript can be published in the EMBO Journal with some minor mandatory revisions.

> We agree with this and the other referees that the functional and structural data were not sufficiently connected in the old manuscript. As pointed out above and most importantly, we now added new data showing that the disome, as it was used for the structural analysis, is also the preferred target of uS10 modification by Hel2 (Fig. 3E). In addition, we made an effort throughout the entire text to connect the functional and structural data in a more proficient way.

Among those, the authors use non-canonical names for protein factors. For example, Rqt1 is used throughout the manuscript but not by others in the field. This name is not even referenced in the database of yeast standard names (SGD)! A situation in which each group uses its own nomenclature is only increasing confusion (e.g., searches on literature or general database will not retrieve all reports characterizing these factor). Authors should use standard gene names throughout their manuscript.

> Thank you for your constructive suggestion. As suggested, we replaced “Rqt1” with Hel2 throughout the text. Rqt4 has been registered in SGD as a standard name. Rqt1-3 have been described as aliases of Hel2, Slh1 and Cue3, respectively, in our previous study (Matsuo et al. 2017).

A second point that could be improved is the presentation of methods, pre-existing data and related work. Often references are indeed missing.

> Thanks for pointing out this shortcoming. We now carefully re-checked the presentation of methods, pre-existing data and related work, and adjusted their presentation accordingly. Moreover, we inserted missing references in the correct positions including Brandman et al. 2012 as also suggested by Referee #3.

For example:
- the method used to prepare the cell free translation extract that served to generate the structural data is not indicated (page 38, last line);

> Thank you for pointing this out (as also noticed by Referee #2). We have modified the Methods section accordingly (page 42, line 34), to increase clarity. This section now reads as follows:

"*His-HA-uL4-(CGA-CCG)$_2$* mRNA was produced using the mMessage mMachine Kit (Thermo Fischer) and used in a yeast cell-free translation extract from *ski2*Δ cells. This yeast translation extract was prepared and *in vitro* translation was performed essentially as described before (Waters & Blobel, 1986). The cells were grown in YPD medium to OD_{600} of 1.5-2.0. Spheroblasts were prepared from harvested and washed cells using 10 mM DTT for 15 minutes at room temperature and 2.08 mg zymolyase per 1 g of cell pellet for 75 minutes in 1 M sorbitol at 30°C. Spheroblasts were then washed and lysed in a Dounce homogenizer as described before (Waters & Blobel, 1986) using lysis buffer comprising 20 mM Hepes pH 7.5, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 10% Glycerol, 1 mM DTT, 0.5 mM PMSF and complete EDTA-free protease inhibitors (GE Healthcare). The S100 fraction of lysate supernatant was passed through PD10 column (GE Healthcare) and used for *in vitro* translation."

- evidence that ski2-E445Q is dominant negative is not indicated (page 10, 3 lines from bottom);

Thank you for this valid point that was also raised by referee #2. We constructed the ski2-E445Q mutant and described the evidence for its dominant-negative activity on *GFP-Rz* mRNA in our previous study (Ikeuchi & Inada, 2016).
Along with your suggestion, we added the reference of our previous study and some new experiments demonstrating its dominant-negative effect in Appendix Figure S8. We could confirm 5'NGD intermediate (A), and synthetic growth defect in *xrn1* deletion cells (B) by overexpression of ski2-E445Q. We also confirmed that ski2-E445Q mutant could not rescue the lethality of *ski2*Δ*xrn1*Δ double deletion cells (C).

- molecular models docked in the EM envelope are not mentioned in the main text (page 13);

Thanks for pointing out this missing information, which was given in the Methods section only. We have used models by Ben-Shem et al., 2010 (PDB ID: 4V88) and Schmidt et al., 2016 (PDB ID: 5MC6) and the proper references were now added to the main text (page 14, lines 21):
"The most stable disome structure adopted the most compact overall conformation permitting a 5.3 Å average resolution and docking of molecular models (Fig 4A-B; Appendix Fig S4, S5A and S6; Schmidt et al., 2016; Ben-Shem et al., 2010)."

- sentences refereeing to previous work of the authors on Rtq1 bound ribosomes do not contain a reference (page 21, line 5), etc.

Thank you for pointing out the missing reference, which we have now inserted (page 24, lines 21):
"Our previous study showed that affinity-purified Hel2-ribosome complexes mainly yielded 80S ribosomes in the rotated state (Matsuo et al., 2017), suggesting that Hel2 may preferentially bind colliding rotated ribosomes as a primary contact site (Fig EV5)."

Careful reading the manuscript to add missing references is thus necessary before publication. Authors should also provide more arguments on the validity of their experiments in the main text. Without going into details of the supplementary data and methods, it is difficult to understand by reading the manuscript whether the disome structure presented is representative of a large fraction of the structures observed by

cryo EM and why it should be considered as informative. These arguments should be presented concisely in the manuscript, particularly in the absence of validating biological data. Indeed, it remains possible that this model represents only an artefactual structure formed in vitro.

We thank the referee for pointing out the mentioned weaknesses in the manuscript, which we addressed as follows:
a) We carefully went through the manuscript and added missing references.
b) Wherever possible, we tried to provide more clarity and described more accurately the rationale and the conclusions of performed experiments, thereby providing more supporting arguments on the validity of our data.
c) For the Cryo-EM part, we were indeed short in describing the procedure in the main text and moved detailed descriptions into the Supplement. In fact, we used widely established methods and no manipulations in order to obtain the disome structure, which represents about one half of the identified particles. The remaining half represents 80S monosomes, that were apparently resulting from disome dissociation during purification and vitrification (as often observed), since we initially collected disome fraction from the sucrose density gradient. All disomes classes observed displayed an essentially identical arrangement with interacting small subunits. We observed a population of these disomes with a very rigid arrangement and a very well defined interface allowing for a relatively well resolved disome structure determination. Taken together, this supports the idea that unique three-dimensional structural features occurring upon ribosome collision could be recognized by Hel2 and/or other quality control factors.
d) In order to present these and additional arguments more precisely, we adjusted the chapter on the structural findings accordingly, and also modified the Appendix Fig. S5 of the Supplement. Moreover, as mentioned above, we performed an additional experiment showing that our mRNA reporter-stalled disomes represent a preferred Hel2 substrate over monosomes (Figure 3E). We thus believe, that it is rather unlikely that this structure would represent an artefact or that it would not have informative value.
Finally, a very recent joint study by the Hegde and Ramakrishnan labs (Juszkiewicz et al., 2018) presented an essentially identical principle in the mammalian system: molecular recognition of stalled disomes by ZNF598 (the mammalian homolog of Hel2) triggers induction of RQC. These findings in the mammalian system are in perfect agreement with ours and therefore fully support our observations in yeast, and suggest a highly conserved pathway.

Finally, the discussion could probably be reinforced e.g., through indications why and how the CCR4-NOT complex could be involved in this process.

We agree, and, as suggested, we augmented the Discussion section by indicating how and why the Ccr4-Not complex may be involved in this process.
Notably, the Ccr4-Not complex plays multiple roles in gene expression, and an important role of Ccr4-Not in deadenylation of the poly-A tail for induction of mRNA decay has been suggested. We have analysed Not4 function, but we have not focused on the entire Ccr4-Not complex in this study, since Not4 alone was sufficient for ubiquitination of eS7 *in vitro*. We have no evidence suggesting that other components of Ccr4-Not function in NGD^{RQC-} *in vivo.* However, it is likely that Not4 may be recruited to the ribosome with the other components of the Ccr4-Not complex, since in yeast they form a stable complex (Collart & Panasenko, 2012). We have inserted (page 26, line 20): "The polyubiquitination of eS7 requires monoubiquitination by Not4, so we suspect that also the stable yeast Ccr4-Not complex (Collart & Panasenko, 2012) could interact with stalled poly-ribosomes. However, it is not clear how

and when Not4 recognizes and ubiquitinates the ribosome and how the activity of Not4 can be related to ribosome collisions. We speculate that other component(s) of the Ccr4-Not complex may serve as selectors of the target ribosome(s) directing Not4 to its sites of action, such as eS7 in the case of NGD^{RQC-}. Further studies will be needed to uncover the regulatory relevance of sequential ubiquitination of eS7 in the determination of mRNA fate."

Accepted 10th December 2018

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by three of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

REFEREE REPORTS.

Referee #1:

The authors have successfully addressed all of my concerns with the initial manuscript. I recommend that the current manuscript be accepted for publication.

Referee #2:

The authors addressed my main concerns and I really appreciate the amount of work they carried out to make the paper much better. Given the way they handled other reviewers' comments and the overall enthusiasm from all the reviewers, I support the publication of the manuscript.

Referee #3:

The authors have satisfied all of my requests. The revised manuscript is an outstanding contribution to our understanding of translational quality control, the role and mechanism of ribosome collisions in initiating quality control, and the functions of the RQT and RQC pathways. I hope to see it published without delay.

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: Toshifumi Inada, Roland Beckmann
Journal Submitted to: EMBO Journal
Manuscript Number: embj2018-100276

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 justified
- 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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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?	NA
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?	NA
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.	NA
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
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5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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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).	This is provided in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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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	The cryo-EM density for the stalled di-ribosome has been deposited in the Electron Microscopy Data Bank with accession code EMD-4427 and in the Protein Data Bank with accession code 6I7O.
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).	All EM reconstructions have been deposited at the above accession codes.
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