Not4-dependent translation repression is important for cellular protein homeostasis in yeast

Steffen Preissler, Julia Reuther, Miriam Koch, Annika Scior, Michael Bruderek, Tancred Frickey and Elke Deuerling

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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.)

Editor: Anne Nielsen

1st Editorial Decision 03 December 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal and my sincere apologies for the unusual delay in the review process in this case. We have now finally heard back from three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript, although they also raise a number of concerns that will have to be addressed in full before they can support publication. In particular, ref #1 (who also saw the earlier submission of this study) finds that the data supporting the model for translational repression as the main role for Not4 remain partially inconclusive and need to strengthened further. This point is reflected in the comments from ref #3 (new ref) who finds that the quantitative aspects of the work needs to be established better and that several reported observations appear at odds with the underlying model. I realize that ref #2 (also new ref) is the most positive of the three, but given the nature of the concerns raised by the other refs (especially concerning the conclusiveness of the data) the points raised by ref#1 and #3 will need to be addressed in full.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.)
Thank you for the opportunity to consider your work for publication and once again sorry for the delay in the review process here. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

The authors have improved the manuscript and included additional data. However some of the comments on the previous manuscript were not addressed.

The manuscript investigates the role of Not4, a subunit of the Ccr4-Not complex, in quality control during protein synthesis.

Although I still think that this a timely and potentially interesting manuscript that provides insight into protein and mRNA quality control mechanisms, I am not entirely convinced by the interpretation of the results. For example, the major effect observed in the absence of Not4 is protein aggregation and a failure to repress global translation upon glucose or amino acid starvation, all other effects are minor and only observed in cells lacking Ltn1. Therefore, it is possible that the major role of Not4 is to facilitate co-translational protein folding or to repress translation globally. Although the role in global translational repression could be indirect through Dhh1. However, the authors conclude that "Not4 represses translation of mRNAs that cause ribosome stalling". The evidence for this conclusion is less compelling because the effects of Not4 depletion for all reporters tested are only seen in cells that also lack Ltn1.

Additional comments:
1. One general comment on the previous manuscript concerned the reproducibility of the results as there was no quantitation. Now the authors have quantified westerns and northerns but no error bars are included in most cases. Given that the effects are relatively small, the authors should indicate how many replicates were obtained.

2. The effects of Not4 on mRNA stability should be confirmed by determining mRNA half-lives. In particular because transcriptional effects cannot be ruled out, given the role of the CCR4-NOT complex in both mRNA degradation and transcription.

3. What is the explanation for the effects observed in cells lacking decapping factors? Are the mRNAs accumulating in these cells decapped? This can be tested using terminator nuclease.

Minor comments:
The abstract should mention that this study was done in yeast. In particular because Not4 is not a stable component of the Ccr4-Not complex in metazoans. Thus, some of the effects described in this manuscript could be specific to yeast.

The authors should mention that Not4 is not a core subunit of the Ccr4-Not complex in metazoan.

On page 5 the authors conclude that Not4 and Ccr4-Not function in translation because they associate with polysomes in an mRNA dependent manner. This conclusion is not warranted. These proteins may associate with translating mRNAs without functioning in translation.

On page 18 the authors cite the manuscript by Ruoya et al. but they ommitted two previous manuscripts showing the interaction of DDX6 with the CCR4-NNOT complex in metazoans: Chen et al., (2014) Mol Cell 54, 737 and Mathys et al., (2014) Mol. Cell. 54, 751.

Referee #2:
The E3 ligase, Not4, has been known to function in ribosome-associated quality control, but its exact role and underlying mechanisms have remained unclear. In this paper, Deuerling and collaborators provide straightforward and convincing evidence that Not4 represses translation of transcripts that cause transient ribosome stalling within open reading frames. In the course of investigating this problem, the authors additionally uncover a role for factors involved in mRNA decapping in this quality control pathway. Finally, the authors report that the absence of Not4 enhances the expression levels of arrested nascent polypeptides and causes constitutive folding stress as well as protein aggregation. In conclusion, Not4 plays a role in quality control by mediating translation repression of certain aberrant transcripts, and this process is critical to maintain proteome integrity. The important discoveries presented in this manuscript will be of interest to researchers studying mRNA and protein quality control, translational regulation, and protein aggregation. The manuscript is clearly written, and experiments are well designed and beautifully executed, as is typical of work from the Deuerling lab. As such, this work is appropriate for publication in EMBO J.

Minor comments

1. Not4 is presumably substoichiometric relative to ribosomes. Thus, there must be a mechanism that allows Not4 to identify and bind to ribosomes engaged in translation of mRNAs that cause transient stalling. Although identification of this mechanism is beyond the scope of the current paper, it would be useful for the authors to suggest plausible mechanisms that might be used. It is intriguing that RNase A treatment collapsed polysomes but failed to displace Not4 from ribosomes. Along these lines, it would be informative to know whether puromycin treatment is able to displace Not4 from ribosomes.

2. Regarding Figs. 3C and 3D, and the paragraph on page 9 beginning with "To reduce the dominant effect...": if one impairs K12 protein degradation by deleting Ltn1, it now becomes apparent that the extent of translational repression attributed to Not4 is similar in K0 and K12 templates; please clarify.

3. The effect of Not4 on translational repression of K12 mRNA proposed by the authors is independent of the RING domain and E3 ligase activity of Not4. Thus, it appears that the phenomenon characterized by the authors is distinct from the Not4 effect on K12 expression reported by Inada and colleagues, which is RING domain-dependent (Dimitrova et al 2009, "Nascent peptide-dependent translation arrest leads to Not4p-mediated protein degradation by the proteasome. J Biol Chem. 17:284"). This needs to be discussed.

4. To this reviewer’s knowledge, an effect of Not4 deletion on the K12 protein degradation rate has not been previously reported. Although not directly relevant for the claims made by the authors in this paper, it would be useful to have this issue directly addressed. (Perhaps such an experiment could be presented along with Fig. E3A.)

Referee #3:

Protein biosynthesis is a multistep process involving the transcription of RNA from DNA sequence, mRNA maturation, the translation of RNA to protein, and the proper folding of the nascent peptide. Errors can occur at each step of this process, and the downstream consequences (usually protein aggregation) are often deleterious. As a result, the cell uses multiple triage pathways to ensure fidelity. Since errors in different stages have different levels of permanency, i.e. a transcription error will affect all translation, the cell must respond appropriately for maximum efficiency. Many players have been recently identified, although the role and mechanism of each factor is poorly understood. Such factors include the extensive Ccr4-Not complex that appears to have multiple roles in minimizing aberrant mRNA and nascent chains. Preissler and colleagues present evidence that a component of this complex, Not4, acts to reduce the production of problematic proteins through repression of elongation, rather than mRNA depletion or protein degradation.
Overall, the authors provide convincing and thorough evidence that Not4 acts through a novel mechanism of translational repression, and given its evolutionary conservation through eukaryotes, this is significant to our understanding of other situations of perturbed translation, such as in cancers and viral infections. The manuscript will be suitable for publication after the following points are addressed.

Please present quantification of 2A-K0 full length levels vs K0 full length levels to confirm that the 2A element is causing a pause as argued in Figure 3D.

Please add error bars on quantifications in 3C and 3D.

It is not so clear that most stalled K12-M polypeptides stay bound to ribosomes in the not4/ltn1 deletion (Figure E2, and bottom of page 6), and it makes more sense that they don't. If RNCs were stalled but stable, and if expression is increased in Not4 deletion, a ladder of products due to back-up should emerge.

The transition from Figure 3 to Figure 4 is confusing with regard to mRNA levels. In Figure 3B, there is minimal change in mRNA level in the strains, while in Figure 4B there are substantial differences. The constructs differ by the 2A element and the location of the K12 stretch. While results in Figure 3B, and Figures 4B and 4C nicely decouple mRNA level from accumulation of truncated nascent chains, the paper would benefit from acknowledging earlier that the two reporter constructs cause different effects of mRNA stability in the different strains. It subtly supports the notion that total mRNA level, expression levels, and Not4 repression operate through distinct mechanism.

How exactly are ribosomes quantified in Figure E6? If it is from mass spec analysis of the aggregates, it is not appropriate as it does not rule out aggregation of ribosomal proteins directly. Also the legends for E6A and E6B are reversed.

Please explain why wild-type polysome profiles have a low polysome to monosome ratio.

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**Point to point answers**

**Referee #1:**

This referee said that “...Although I still think that this a timely and potentially interesting manuscript that provides insight into protein and mRNA quality control mechanisms, I am not entirely convinced by the interpretation of the results. For example, the major effect observed in the absence of Not4 is protein aggregation and a failure to repress global translation upon glucose or amino acid starvation, all other effects are minor and only observed in cells lacking Ltn1. Therefore, it is possible that the major role of Not4 is to facilitate cotranslational protein folding or to repress translation globally.”

**Authors:** We do not rule out a function of Not4 in cotranslational protein folding or degradation. However, that far none of our results directly points into this direction. Additional experiments are required to address this interesting questions which are, however, not within the scope of this study.

We fully agree with the referee that our data show besides specific translation repression of stalled model peptides also a “global” misregulation of translation under starvation conditions and in sum this may provide the explanation for the severe imbalance of protein homeostasis in absence of Not4. To make this aspect more clear we introduced the following changes in the manuscript:

- We changed the title of the article to “Not4-dependent translation
repression is important for cellular protein homeostasis in yeast”.

• We do not use the expression “mRNA-specific” in the abstract when describing the function of Not4 in translation repression.

• We include the “global” effects of NOT4 deletion in the abstract by saying: “The absence of Not4 affected global translation repression upon nutrient withdrawal, enhanced the expression levels of arrested nascent polypeptides and caused constitutive protein folding stress and aggregation.”

• We changed the description of Not4’s role in “quality control on translating ribosomes” in the abstract and more precisely describe its function as “regulation of protein expression on the mRNA level”.

• We now say at the beginning of the discussion: “The defects in overall translation repression upon nutrient withdrawal as well as severe translation-dependent protein folding stress in the absence of Not4 suggest that Not4 (and probably other components of the Ccr4-Not complex) plays a rather general role in translation repression.”

• In addition we rephrased the headline of the paragraph describing translation repression in response to nutrient withdrawal (line 291): “Not4 and decapping proteins are required for fast global translation repression upon nutrient withdrawal”

• The first sentence of the paragraph describing the effects of NOT4 deletion on proteome integrity may have been misleading as it focuses too much on the prevention of the synthesis of “defective and potentially harmful proteins”. We rather say more generally “Regulation of protein synthesis and cotranslational quality control are critical to facilitate the coordinated supply of new and functional proteins according to cellular demand”

We do not agree with the reviewer that the effect of Not4 on the expression of arrested nascent polypeptides are “minor” for the following reasons:

• The use of model proteins such as ribosome stalling reporter constructs is a valuable tool used before by many others (Bengtson & Joazeiro, 2010; Chiabudini et al, 2012; Dimitrova et al, 2009; Ito-Harashima et al, 2007; Tsuboi et al, 2012) to analyze and dissect the effect of quality control factors on the level of individual mRNAs.

• The fact that deletion of LTN1 is required to uncover the Not4 effect draws on intrinsic features of eukaryotic cotranslational protein quality control and the ability of this system to adjust to the loss of Not4 but does not argue against the significance of Not4 for this pathway. Loss of Not4 clearly causes enhanced expression of damaged and arrested nascent polypeptides, however, these polypeptides become rapidly degraded via Ltn1 ubiquitination. This assumption is confirmed by the fact, that we found more Ltn1-dependent ubiquitination of the reporter 2A-K12 in not4Δ cells (see Fig. 3E).

Reviewer #1: … the role in global translational repression could be indirect through Dhh1. However, the authors conclude that “Not4 represses translation of mRNAs that cause ribosome stalling”. The evidence for this conclusion is less compelling because the effects of Not4 depletion for all reporters tested are only seen in cells that also lack Ltn1.

Authors: It is currently not understood how Dhh1 and the decapping factors repress translation mechanistically. However, we agree with the reviewer that Not4 may function in translation repression through the action of Dhh1 and the decapping factors. Our data support this notion and we propose this in the text. The model which we provide is also based on this assumption (Figure 9). We admit that saying “Not4 represses translation” suggests an active process for which we have no direct evidence yet and therefore might be misleading. We do not use this expression anymore. Instead the summary of our findings at the end of the introduction now reads as follows:

“Here we demonstrate that Not4 plays a crucial role in cotranslational quality control, however, it does not directly contribute to the ubiquitination and turnover of arrested nascent polypeptides. Instead, our data indicate that Not4 is required for global translation repression under nutritional limitations and especially for repression of mRNAs that cause transient ribosome stalling. This function likely involves the decapping components Dhh1 and Dep1. Thus, Not4-dependent translation repression adds an additional level of co-translational quality control important for the maintenance of cellular protein homeostasis.”

Reviewer #1: Additional comments:
1. One general comment on the previous manuscript concerned the reproducibility of the results as there was no quantitation. Now the authors have quantified westerns and northerns but no error bars are included in most cases. Given that the effects are relatively small, the authors should indicate how many replicates were obtained.

Authors: Representative Northern und Western blots are shown throughout the manuscript and of course the data have been reproduced in independent experiments as it is the rule of good scientific practice in our lab!

We now quantified the data and provide mean values +/- SD from at least three independent experiments. For better illustration and comparison of data between figures, bar graphs were included where appropriate. We believe that the quantitative analyses further increase confidence and strengthen our interpretations. Quantification has been performed on the following figures:

Figure 2
“B” and “C” show representative blots. Independent experiments were quantified and the data are now presented in a separate bar graph in Figure 2D.

“E”, “F” and “G” show representative blots and quantification of different experiments is now shown under each blot.

Figure 3
“B” shows a representative blot and quantification of different experiments is now shown underneath.

In “C” and “D” the bar graphs under the blots now show mean values +/- SD of three experiments.

Figure 4
In “A” a bar graph was added showing K12-M and K0 mRNA levels +/- SD.
In “B” quantification of independent experiments is now presented for K12-M as well as K0 mRNA levels under representative blots.

Figure 6
In “B”, “C” and “D” bar graphs were included showing K12-M arrest product levels based on data from independent experiments, respectively.

Additional repeats of the experiment shown in “B” were performed and mean values +/- SD of independent experiments are shown.

We also analyzed K12-M mRNA levels in ln1Δdh1Δ cells and ln1Δdcp1-34 mutants, which we have not done before (Figure 6E). This left the possibility that increased arrest product levels in these strains are due to higher reporter mRNA levels. The new data now show that the K12-M mRNA levels were only slightly elevated in ln1Δdcp1-34 cells but not in ln1Δdh1Δ cells. Thus, the strongly elevated K12-M arrest product levels are not due to profound differences in mRNA levels. This important control experiment further supports that Dhh1 and Dcp1 are required for translation repression of ribosome-stalling mRNAs and thus share this function with Not4.

Reviewer #1: 2. The effects of Not4 on mRNA stability should be confirmed by determining mRNA half-lives. In particular because transcriptional effects cannot be ruled out, given the role of the CCR4-NOT complex in both mRNA degradation and transcription.

Authors: We thank the reviewer for this comment. We would like to point out that the main conclusion of Figure 4 is that the observed increase of translation arrest product levels in absence of Not4 and Ltn1 (Figure 2) cannot merely be attributed to an increase in the steadystate levels of the reporter mRNAs. However, the experiments presented in Figure 4 A and B revealed slightly elevated K12-M mRNA levels in not4Δ and not4Δln1Δ cells. These increased levels may contribute to increased expression of the arrested polypeptides but the quantitative discrepancies indicate that altered mRNA levels have only a minor influence on arrest product levels. This is supported by the comparative data shown in Figure 4C: the loss of Ccr4 and Not4...
elevated the mRNA of K12-M in a similar manner but deletion of CCR4 enhanced expression of the arrest product only slightly in contrast to deletion of NOT4.

It is indeed possible that the slightly (1.5-to-2-fold) increased K12-M mRNA levels in absence of Not4 can be attributed to reduced turnover rates. As suggested by the referee, we analyzed mRNA half-lives of the K0 and K12-M reporter mRNAs in presence and absence of Not4 and present the data in an additional supplementary figure (Figure E5). Based on the data we draw the following conclusions: First, small changes in the nucleotide sequence of the reporter mRNAs can indeed influence their stability and decay rates (as stated by reviewer 3, see below). In this case, the half-life of the K12-M mRNA (5.4 min) was slightly longer than the half-life of the K0 mRNA (3.8 min) in wild-type cells, although both constructs differ only in the K12-encoding sequence. Second, the half-lives of both, the K0 and K12-M mRNA were slightly increased in absence of Not4. This suggests that mRNA turnover is indeed moderately affected in not4Δ cells, however, the differences to wild-type cells were statistically not significant. Based on these data the increase of K12-M mRNA levels in absence of Not4 can indeed be partially due to elevated mRNA half-lives. Interestingly, it has been shown recently that during microRNA-mediated repression in mammalian cells the target mRNAs are first translationally repressed, which is a prerequisite for enhanced target mRNA decay. In that case mRNA decay seems to be a consequence of translation repression. It is therefore possible that Not4-dependent translation repression may to some extent also influence mRNA decay rates [see (Meijer et al., 2013)]. We added this reference to our discussion (line 479): “More recently translation repression during microRNA (miRNA)-mediated gene silencing in human cells was shown to be an independent process required for subsequent mRNA destabilization (Meijer et al., 2013). This involves the translation initiation factor eIF4A2, which also interacts with the Ccr4-Not complex.” We also included “mRNA decay” as a potential downstream effect of translation repression into our model (Figure 9) to account for this point of the reviewer’s criticism.

It is also worth to point out here that the Ccr4-Not complex has previously been functionally connected to translation repression e.g. during microRNA-mediated gene silencing in mammalian cells (see references in the discussion). The mechanism by which this occurs is not well understood yet. In contrast, in yeast, the complex was mainly associated with regulation of mRNA expression and many observed effects were primarily attributed to its role in mRNA turnover. Our experiments distinguish between these processes and support that the Ccr4-Not complex functions in translation repression also in yeast. This also suggests that the function of the Ccr4-Not complex in translation repression is evolutionarily conserved [we mention this in the discussion (line 491): “The principal function of Ccr4-Not components in translation repression seems thus to be conserved”]. While in higher eukaryotes this function may have become more specialized (e.g. microRNA-mediated repression) it seems more global in yeast. These findings may thus inspire further studies on the conserved basic mechanism of translation repression in eukaryotes.

Review #1: 3. What is the explanation for the effects observed in cells lacking decapping factors? Are the mRNAs accumulating in these cells decapped? This can be tested using terminator nuclease.

Authors: We thank the reviewer for suggesting this very nice experimental strategy, which we were not aware of before. The question of how Dhh1 and the decapping enzymes (and Not4) contribute mechanistically to translation repression and if the removal of the 5’ cap as such is crucial for this function is indeed an interesting open question. As decapping and mRNA decay are thought to be intimately coupled processes, it is technically very challenging to reliably detect decapped mRNAs in vivo.

The reviewer asked in particular whether decapped mRNAs accumulate in mutant cells. However, it is difficult to envision how decapped mRNAs could accumulate if decapping may be impaired. We rather would expect the opposite effect when factors involved in decapping are missing or mutated, namely the accumulation of capped mRNAs due to reduced translation repression and mRNA turnover. On the other hand, the question whether decapped mRNAs accumulate in wild-type cells, where translation repression is functional, is warranted. This may
shed light on the requirement of decapping as such to repress translation of the transcripts. As we agree with the reviewer that the use of terminator nuclease would offer such an experimental approach we performed the suggested experiment.

We compared the non-stalling K0 and the stalling K12-M mRNA levels in wild-type and not4Δ cells. As a control we included dcp1-34 cells, which have a described decapping defect (Tharun & Parker, 1999). The result of a representative experiment is shown below for inspection by the referee.

We noticed an increase in the Northern blot signals when terminator nuclease was present for which we have no explanation. This effect has been observed in another study as well (Braun et al, 2012). Consistent with our other results the K12-M mRNA levels were slightly higher in not4Δ cells. In dcp1-34 mutants the mRNA levels were further elevated, which agrees with Dcp1 acting in mRNA decapping and decay. However, we found that both mRNAs were insensitive to terminator nuclease-treatment in either of the genetic backgrounds, indicating no detectable accumulation of decapped mRNAs. This is likely due to the rapid degradation of decapped mRNAs before and during cell lysis. We think that performing such an experiment in another genetic background (which is currently not available) where the 5’ exonuclease Xrn1 is absent to slow down degradation of decapped mRNAs would be a reasonable setup. This is, however, timely and conceptually beyond the scope of the present manuscript and will be addressed in a separate study that will focus on the mechanistic details.
Figure: The experiment was performed according to (Braun et al, 2012). Wild-type (wt) and the indicated yeast strains transformed with plasmids expressing either non-stalling K0 reporter mRNA or the ribosome-stalling K12-M mRNA and total RNA was isolated by hot phenol extraction and ethanol precipitation. RNA samples (25 µg) were digested in the presence or absence of 4 U Tobacco Acid Pyrophosphatase (CapClip Acid Pyrophosphatase, Cellscript) in a 50 µl reaction for 2 h at 37 °C as recommended by the manufacturer. After phenol/chloroform/isoamyl- (Roti-Aqua-PCI, Roth) extraction and ethanol precipitation equal amounts of the RNA samples (6-7 µg) were incubated with or without 1 U Terminator 5'-phosphate-dependent exonuclease (Epicentre) in a 20 µl reaction volume for 1 h at 30 °C as recommended by the manufacturer. Each reaction was supplied with three nanograms of an in vitro transcribed 5' triphosphate RNA (spike) to control for the specificity of the Terminator exonuclease. Equal amounts (final 3 µg) of each sample were used for Northern Blotting. Ribosomal RNA (rRNA) was detected by methylene blue (MB) staining of the membrane. Bar
graph: The reporter mRNA signals were quantified and normalized to the spike RNA signal.
Very similar results were obtained in an independent experiment.

Based on the reviewer’s comment we also realized that the potential relationship between Not4-mediated translation repression and “decapping” may not have been clearly presented. We thank the reviewer for making us aware of this shortcoming. In a narrow sense removal of the 5’ cap primarily leads to mRNA degradation rather than translation repression. However, it is well accepted that the process of decapping is intimately linked to translation repression via modulation of translation initiation. For example, translation initiation factors that bind to the 5’ cap structure have been shown to prevent decapping whereas their dissociation seems to mark the exit of an mRNA from translation, which precedes its decapping and decay. Decapping as such may thus not be the primary event of translation repression (but could rather be a consequence). Therefore, Not4 as well as the decapping factors may facilitate translation repression not by directly stimulating the removal of the 5’ cap but by decapping-related mechanisms that inhibit translation initiation. We modified the text especially in the discussion and the description of our proposed model and provide further important references to make this point clear. For example we now say in the discussion: “Translation initiation and mRNA decapping are reciprocal events. Accordingly, decapping can be directly inhibited by cap-binding translation initiation factors which dissociate from transcripts before decapping occurs (Schwartz & Parker, 1999; Schwartz & Parker, 2000; Tharun & Parker, 2001). Although the mechanism for the latter process is still unclear it likely marks the exit of an mRNA from translation before its degradation. Thus, Not4-dependent translation repression may occur more directly on the level of translation initiation e.g. by inhibition or displacement of an initiation factor. Importantly, in vitro experiments suggest that Dhh1 primarily represses translation initiation, which then indirectly promotes decapping (Coller & Parker, 2005; Nissan et al, 2010), whereas other data suggest that Dhh1 rather inhibits elongation (Sweet et al, 2012). More recently translation repression during microRNA (miRNA)-mediated gene silencing in human cells was shown to be an independent process required for subsequent mRNA destabilization (Meijer et al, 2013). This involves the translation initiation factor eIF4A2, which also interacts with the Ccr4-Not complex.”

The legend for Figure 9, which depicts the proposed model, reads now: “Figure 9: Translation repression of ribosome-stalling mRNAs involves Not4, Dhh1 and the decapping factors Dcp1-Dcp2. Not4, together with the Ccr4-Not complex, associates with polysomes (grey) that likely contain stalled (red cross) and jammed ribosomes. Transient ribosome stalling on mRNAs within open reading frames (ORF) leads to Not4-dependent translation repression. The decapping activator Dhh1 as well as the decapping proteins Dcp1-Dcp2, which remove the 7-methylguanosine (m7Gppp) cap structure from the 5’ end of mRNAs, are also required for translation repression of ribosome stalling mRNAs, suggesting that the Ccr4-Not complex and the decapping machinery act together in this process. Potential repression mechanisms include modulation of transcript-specific decapping or direct inhibition of translation initiation. This prevents further ribosome jamming and synthesis of arrested proteins. Upon disassembly of stalled ribosomes the arrested nascent chains (NC) are ubiquitinated by Ltn1 to initiate their degradation and mRNAs may become eliminated.”

Reviewer #1: Major comments:
The abstract should mention that this study was done in yeast. In particular because Not4 is not a stable component of the Ccr4-Not complex in metazoans. Thus, some of the effects described in this manuscript could be specific to yeast.

Authors: We clearly mention in the abstract and the title that we investigate the role of Not4 in yeast.

Reviewer #1: The authors should mention that Not4 is not a core subunit of the Ccr4-Not complex in metazoan.

Authors: We now mention in the discussion that Not4 is not a component of the Ccr4-Not core complex in metazoans and point out potential functional differences: “Interestingly, Not4 seems not to be a core subunit of the Ccr4-Not complex in metazoans (Lau et al, 2009; Temme et al, 2010), suggesting potential functional and mechanistic differences.”
Reviewer #1: On page 5 the authors conclude that Not4 and Ccr4-Not function in translation because they associate with polysomes in an mRNA dependent manner. This conclusion is not warranted. These proteins may associate with translating mRNAs without functioning in translation.

Authors: We agree with the reviewer that the interaction between the Ccr4-Not complex and translating ribosomes may as well occur via an interaction with mRNA. Mild RNase treatment in Figure 1B efficiently converted polysomes into monosomes. However, these monosomes may still carry larger mRNA fragments via which binding of Not4 and the Ccr4-Not complex may occur. As puromycin disassembles ribosomes and releases nascent chains as well as mRNA we specify our conclusion by saying (line 131) “Moreover, the association of Not4 and Caf1 with ribosomal particles was lost upon ribosome disassembly by puromycin treatment, indicating that the Ccr4-Not complex interacts specifically with assembled (poly-) ribosomes carrying nascent polypeptides and mRNA (Figure 1C).”

Reviewer #1: On page 18 the authors cite the manuscript by Ruoya et al. but they ommitted two previous manuscripts showing the interaction of DDX6 with the CCR4-NNOT complex in metazoans: Chen et al., (2014) Mol Cell 54, 737 and Mathys et al., (2014) Mol. Cell. 54, 751.

Authors: We now cite both studies in the discussion.

Referee #2: This referee states that “The manuscript is clearly written, and experiments are well designed and beautifully executed, as is typical of work from the Deuerling lab. As such, this work is appropriate for publication in EMBO J.”

Reviewer #2: Minor comments
1. Not4 is presumably substoichiometric relative to ribosomes. Thus, there must be a mechanism that allows Not4 to identify and bind to ribosomes engaged in translation of mRNAs that cause transient stalling. Although identification of this mechanism is beyond the scope of the current paper, it would be useful for the authors to suggest plausible mechanisms that might be used. It is intriguing that RNase A treatment collapsed polysomes but failed to displace Not4 from ribosomes. Along these lines, it would be informative to know whether puromycin treatment is able to displace Not4 from ribosomes.

Authors: Not4 is indeed expressed at relatively low levels and our data indicate that Not4 associates especially with large polysomes. This suggests a kinetically driven mechanism that allows fast sampling of ribosomes. As shown in Figure 1C, puromycin treatment indeed largely displaces Not4 from ribosomes. We now include these aspects and discuss possible mechanisms in our discussion:

“For example in metazoans, the translation repressor protein FMRP, which shows a similar distribution in polysome profiles as Not4 (Darnell et al, 2011), binds first to its target mRNAs and later during translation directly to ribosomes, where it likely inhibits elongation through steric effects (Chen et al, 2014). The FMRP-ribosome interaction involves RNA-binding motifs. Not4 contains also a RNA recognition motif (RRM) raising the possibility of a similar mode of interaction. However, unlike FMRP, Not4 dissociates from ribosomes upon their disassembly with puromycin (Figure 1C), indicating different interaction characteristics.”

Reviewer #2: 2. Regarding Figs. 3C and 3D, and the paragraph on page 9 beginning with "To reduce the dominant effect...": if one impairs K12 protein degradation by deleting Ltn1, it now becomes apparent that the extent of translational repression attributed to Not4 is similar in K0 and K12 templates; please clarify.

Authors: This paragraph has been rephrased to avoid confusion and changes will be explained below in the section of reviewer 3.

Reviewer #2: 3. The effect of Not4 on translational repression of K12 mRNA proposed by the authors is independent of the RING domain and E3 ligase activity of Not4. Thus, it appears that the phenomenon characterized by the authors is distinct from the Not4 effect on K12 expression
reported by Inada and colleagues, which is RING domain-dependent (Dimitrova et al 2009, "Nascent peptide-dependent translation arrest leads to Not4p-mediated protein degradation by the proteasome. J Biol Chem. 17:284"). This needs to be discussed.

Authors: At the beginning of the study we were unable to reproduce the effect on K12 protein expression reported by Dimitrova et al. (Dimitrova et al, 2009). One explanation may be yeast strain-specific effects as a different yeast strain was used by Dimitrova et al. [W303 whereas in this study a S288C derivative (BY4741) was used] and cotranslational protein degradation may not be equally efficient so that accumulation of arrest products could be detected even in presence of Ltn1. We also realized that the full-length K12 reporter construct may sometimes be cleaved and the cleavage products can be easily misinterpreted as arrest products (whose stability may be different in different knock-out cells).

To circumvent this problem we first identified the translation arrest products of the K12-M construct by probing the Western blots with antibodies against GFP (before the arrest sequence) and with antibodies against the Flag-tag (behind the arrest sequence). As shown in Figure 2B the arrest product appears only on the GFP blot whereas degradation products of similar size are visible on both blots. Without this initial control experiment degradation and arrest products can easily be confused. Dimitrova et al. do not present a Flag blot to show this specificity. In addition, our observation fully agrees with the observation made by (Bengtson & Joazeiro, 2010) and potential reasons for the different observations were discussed by these authors in detail.

Reviewer #2: 4. To this reviewer's knowledge, an effect of Not4 deletion on the K12 protein degradation rate has not been previously reported. Although not directly relevant for the claims made by the authors in this paper, it would be useful to have this issue directly addressed. (Perhaps such an experiment could be presented along with Fig. E3A.)

Authors: Figure E3 addresses the influence of Not4 on the stability of K12 arrest products by comparing ltn1Δ and not4Δltn1Δ cells. We could not detect significant differences suggesting that the turnover of newly-synthesized proteins is not significantly altered in absence of Not4. It is therefore unlikely that the increase of arrest product levels in not4Δltn1Δ cells compared to ltn1Δ is caused by profound differences in arrest product stability.

Referee #3:
This referee said: “Overall, the authors provide convincing and thorough evidence that Not4 acts through a novel mechanism of translational repression, and given its evolutionary conservation through eukaryotes, this is significant to our understanding of other situations of perturbed translation, such as in cancers and viral infections. The manuscript will be suitable for publication after the following points are addressed. Please present quantification of 2A-K0 full length levels vs K0 full length levels to confirm that the 2A element is causing a pause as argued in Figure 3D. “
Please add error bars on quantifications in 3C and 3D.

Authors: We quantified Figure 3C and 3D and provide SD bars based on data from three independent experiments. In addition, we rephrased the paragraph describing the experiments performed with the 2A-K12 and 2A-K0 constructs to make the conclusions clearer.

We draw the following main conclusions from Figures 3C and 3D: First, and most importantly, the absence of arrested 2A-K12 full-length signal in wild-type and not4Δ cells is consistent with the observations made with the K12-C construct (Figure 2C), which lacks the 2A sequence. At the same time, we observe signals of cotranslationally released GFP-2A in wild-type as well as not4Δ cells. This clearly indicates expression of 2A-K12 in both strains and confirms the notion based on the previous experiments that K12-arrested reporter proteins are subject to Ltn1-mediated cotranslational protein quality control. Second, we also observed a significant increase of released GFP-2A in not4Δ and not4Δltn1Δ cells. This was similar for both, the 2AK12 and 2A-K0 constructs. As the 2A sequence also induces transient ribosome pausing we concluded that GFP-2A expression may also be subject to Not4-dependent translation repression. In this case one would predict that expression of full-length 2A-K0 is influenced by the effect of the 2A sequence. Indeed, the 2A-K0 levels appeared elevated in the absence of
Not4 similar to the GFP-2A levels (Figure 3D). This is not due to altered 2A-K0 mRNA levels as these are similar in all strains (Figure 3B).

To clarify the effect of 2A reviewer 3 asked to compare 2A-K0 and K0 full-length levels in the different yeast strains. We thus quantified K0 full-length levels from several independent experiments and present these data in Figure 2D (grey bars).

Reviewer #3: It is not so clear that most stalled K12-M polypeptides stay bound to ribosomes in the not4/ltn1 deletion (Figure E2, and bottom of page 6), and it makes more sense that they don't. If RNCs were stalled but stable, and if expression is increased in Not4 deletion, a ladder of products due to back-up should emerge.

Authors: We thank the reviewer for addressing this point. Indeed, we never saw significant signals (e.g. a ladder) of fragments smaller than the expected arrest products. We point out that our data are consistent with several previous publications. For example, monosome- and polysome-bound luciferase carrying a C-terminal polylysine-tag was detected as a single band in the total ribosomal fraction and even in polysome fractions of sucrose gradients [Figure 3 in (Chiabudini et al, 2012)]. K12 proteins were also detected mainly as single bands in yeast cell lysates and polysome fractions (Bengtson & Joazeiro, 2010). The reason for this is unclear. One could speculate that only the first ribosome encounters the K12 sequence and becomes tightly stalled. Thus, only the first ribosome would carry the K12 sequence in the tunnel, which may keep the nascent chain within the tunnel due to the electrostatic interactions. The subsequent ribosomes may more easily lose the nascent chains, as these ones are not K12-arrested.

In contrast, we rarely observed larger arrest products. Examples can be seen for the Rz construct in Figure 2G or the K12-M construct in Figure 6B. This observation is consistent with a very recent report from Shen et al. who found mRNA-independent elongation of arrested nascent chains after stalling-mediated 40S disassembly (Shen et al, 2015). In that case, Rqc2, a component of the Ltn1-RQC complex, directly recruits alanine- and threonine-charged tRNAs to the ribosomal A-site, which leads to further elongation and C-terminal tagging of the arrested nascent polypeptide with alanine and threonine extensions (so-called “CAT-tails”). The released arrest products with CAT-tails may then provide an activating signal for heat-shock factor 1 (Hsf1). Interestingly, such an elongated arrest product was also observed in the experiment shown in Figure E2. While the faster migrating band remained partially associated with ribosomes in not4/ltn1A cells the larger arrest product was fully released. This agrees well with the model proposed by Shen et al., however, as the appearance of larger arrest products was not observed in all our experiments (and we did not investigate if the product is “CAT”-tagged) we do not discuss this aspect in the manuscript.

Reviewer #3: The transition from Figure 3 to Figure 4 is confusing with regard to mRNA levels. In Figure 3B, there is minimal change in mRNA level in the strains, while in Figure 4B there are substantial differences. The constructs differ by the 2A element and the location of the K12 stretch. While results in Figure 3B, and Figures 4B and 4C nicely decouple mRNA level from accumulation of truncated nascent chains, the paper would benefit from acknowledging earlier that the two reporter constructs cause different effects of mRNA stability in the different strains. It subtly supports the notion that total mRNA level, expression levels, and Not4 repression operate through distinct mechanism.

Authors: We thank the reviewer for making us aware of this confusing aspect. As described in the response to reviewer 1 (see above) we now included Figure E5, which addresses the effect of loss of Not4 on mRNA half-lives. In addition, we now mention explicitly in the text that small differences in the reporter mRNA sequence can cause differences in mRNA stability and levels in the different strains. “The mRNA half-lives of both, the K0 and K12-M mRNAs, were moderately elevated in absence of Not4, which may explain the increased levels of the K12-M mRNA in not4A cells (Figure E5 and 4A). This also indicates that minimal sequence changes, such as introduction of the K12-encoding sequence, can cause differences in mRNA stabilities and levels in the different strains.”

Reviewer #3: How exactly are ribosomes quantified in Figure E6? If it is from mass spec
analysis of the aggregates, it is not appropriate as it does not rule out aggregation of ribosomal proteins directly. Also the legends for E6A and E6B are reversed.

Authors: The data in Figure E6 (now E7!) were generated exactly as described in the “Expanded view” methods section. We point out that the number of ribosomes was determined exclusively by bioinformatics analysis and is not based on identification of aggregated ribosomal proteins. Instead, the full-length sequences of the proteins identified by mass spectrometry were retrieved from the NCBI database. The corresponding cDNA sequences were then retrieved from the yeast genome database (SGD) and analyzed as described. The ribosome counts were then taken for each sequence from data published in (Arava et al, 2003) for the yeast strain BY4741 (derivatives of which were used in this manuscript). To make this point clear we now say in the results section of the manuscript: “In addition, sequence comparison with genomewide mRNA translation profile data (Arava et al, 2003) revealed that the mean number of ribosomes associated with mRNAs of aggregated proteins was elevated (7 for the aggregated fraction vs. 5 for the non-aggregated fraction; Figure E7B).”

Reviewer #3: Please explain why wild-type polysome profiles have a low polysome to monosome ratio.

Authors: The presented polysome profiles are characteristic for our yeast strains used in this study and the observed conditional differences are highly reproducible. We emphasize that we do not make conclusions based on the absolute monosome-to-polysome ratios as these may be influenced by a variety of factors (e.g. growth media and conditions or strain-specific differences in overall translation activity). More importantly, technical aspects have a major influence on maintenance of polysomes during the experiment and the appearance of the profiles (e.g. reagents, preparation protocol, the method for harvesting the cells, the choice of the sucrose gradient and the equipment used for fractionation). We therefore focus only on relative changes between different conditions and mutants that are reliably detectable with our equipment and preparation protocol. The observed absolute monosome/polysome ratio should thus not influence the interpretation of our results. Additionally, the observed monosome/polysome ratio is not unusual and similar observations were made in other studies (e.g. (Coller & Parker, 2005; Koplin et al, 2010)). Similar monosome/polysome ratios were also shown in (Bengtson & Joazeiro, 2010) where derivatives of the same yeast strain were used. Please note that polysome signals in nutrient withdrawal experiments (Figure 5) were generally lower (compared to the ones observed in Figure 1), which is due to the additional centrifugation and resuspension steps required for medium exchange shortly before addition of cycloheximide and final harvesting (see “Expanded view” methods part). This may reduce overall translation rate but we see no technical alternative for efficient medium exchange.

References mentioned in the point to point answers:


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Thank you for submitting your revised manuscript to The EMBO Journal and my apologies for the slight delay in our reply to you. We have now heard back from two of the original referees and their comments are shown below.

As you will see they both find that the main criticisms have been sufficiently addressed and recommend the manuscript for publication, pending minor revision/clarification as pointed out by ref #1. In addition to addressing these remaining concerns, I would ask you to address the following editorial points in a final revision of your study:

- We generally require that all information relevant to the main experiments in the manuscript should be included in Materials and Methods. I would therefore ask you to move the supplemental materials (at least the experimental sections) into the main manuscript file.

- Concerning the specific points raised by ref #1 I would suggest that you leave the data in fig 1 and 5 in the manuscript, but that you provide further quantitative measures for the conclusiveness of fig 5C.

- In addition, I noticed that a few recent studies addressing the relative contribution from Not4 and CCR4 in quality control might be relevant to briefly mention in your paper (Matsuda 2014, Halter 2014).

- We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Please feel free to contact me with any questions. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to receive the final revision of your manuscript.

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REFEREE COMMENTS

Referee #1:

The authors have revised and improved the manuscript and addressed most of the reviewer's concerns.

I have only a few additional minor comments.

1. Figure 1 could be supplemental. Regarding this figure it is unclear why the distribution of CAF1 in the polysome profile is so different in panels A and B (control). In principle, these panels should be very similar. I suggest to delete Caf1 if it is not reproducible or to confirm the result.

2. Figure 3D. Some bars lack error bars.

3. Figure 5C. The data obtained upon amino acid starvation is not convincing. There is hardly any difference for the not4 delta strain. In contrast the differences obtained upon glucose starvation are clear. Therefore I suggest to delete figure 5C, which is not compelling and not necessary.

4. Page 18, line 470. Translation initiation and decapping are not reciprocal but competing processes.
5. Page 18, lines 479-483. The discussion regarding the Meijer et al. paper should be deleted because the findings reported in this paper could not be reproduced in subsequent studies. First, eIF4A2 does not interact with the CCR4-NOT complex as shown by Chen et al. 2014b, Mathys et al. 2014 and also Rouya et al., 2014. Secondly, eIF4A2 is not recruited but displaced from repressed mRNAs (Fukao et al.2014, Fukaya et al.,2014). If the authors refer to the Meijer et al. paper they should also state that many other papers reached different conclusions, to provide an accurate view of the literature. However, this discussion is not relevant to this paper and therefore it is easier to delete any reference to the Meijer et al. paper. Furthermore mRNA destabilization can occur cotranslationally as shown by Hu et al.(at least in yeast).

Referee #3:

The manuscript is much improved, and the authors have addressed all my concerns. I am happy to accept it.

2nd Revision - authors' response 07 April 2015

Point-by-point response

Response to Editorial points:

Editorial point: We generally require that all information relevant to the main experiments in the manuscript should be included in Materials and Methods. I would therefore ask you to move the supplemental materials (at least the experimental sections) into the main manuscript file.

Authors: We have moved the method part most relevant to the experiments into the main manuscript text.

Editorial point: Concerning the specific points raised by ref#1 I would suggest that you leave the data in fig 1 and 5 in the manuscript, but that you provide further quantitative measures for the conclusiveness of fig 5C.

Authors: As you suggested, we left Figure 5C in the manuscript and performed a quantitative analysis of the polysome profiles to better visualize impaired reduction of polysomes upon amino acid withdrawal (which reflects a defect in translation repression) in absence of Dhhl or Not4. The result of quantification of the profiles shown in Figure 5C and data from two additional independent experiments are presented in two new graphs shown as Figure 5D. The first graph shows the changes of the quantitative distribution of total ribosome particles amongst different ribosome species in presence and absence of amino acids. The graph below shows only the reduction of polysomes upon amino acid withdrawal (as % of polysomes in presence of amino acids). The original profiles used for the quantitative analysis will be provided as "source data" to Figure 5C and D.

The paragraph describing Figure 5C was modified accordingly and reads now:

"Glucose depletion caused the rapid conversion of polysomes into 80S monosomes in wild-type cells, reflecting severe reduction of translation activity (Figure 5A). In contrast, residual polysome peaks were still detected in dhhlΔ mutants after glucose withdrawal, which agrees well with the reported defect in translation repression. Importantly, cells lacking Not4 showed a similar defect (Figure 5A) and the relative rate of protein synthesis upon glucose depletion was higher in not4Δ mutants than in wild-type cells (Figure 5B). Translation repression in dhhlΔ and not4Δ cells was also affected shortly after amino acid withdrawal as evident by the smaller decrease of polysomes (Figure 5C and D). These data suggest that Dhhl and Not4 are both important for fast
translation repression during nutrient starvation.”
We also mention in the Methods section how polysomes profiles were analysed: “... The absorbance data were processed with PeakTrak V1.1 (Teledyne Isco, Inc.) and ribosome species were quantified by calculating the area under the absorbance curve.”
We agree to leave the Caf1 blot in Figure 1 because the distribution of Caf1 in polyosome fractions is highly reproducible. The antibodies directed against Caf1 which we used for Western Blotting are difficult to handle because they do show high specificity but low reactivity/affinity causing variations in the signal intensities in Western Blots. As an example for reproducibility of the Caf1 distribution please see the result of another independent experiment below:

Editorial point: In addition, I noticed that a few recent studies addressing the relative contribution from Not4 and CCR4 in quality control might be relevant to briefly mention in your paper (Matsuda 2014, Halter 2014).

Authors: We cite now both studies by Matsuda et al. 2014 and Halter et al. 2014 in the introduction.

Editorial point: We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Authors: We provide source data of the representative gels and blots shown in the main figures. The relevant source data are assembled in one PDF file per main figure and the blots (including the markers) are labeled. To show the reproducibility of our results we additionally provide data from repeats of the experiments, which are not shown in the main figures but were used e.g. for quantifications. The repeat blots often contain additional lanes with data not relevant for the final manuscript and the order of sample loading may differ in some cases. We therefore labeled all lanes but highlighted the relevant ones.

Responses to concerns of Referee #1:

Referee 1: Figure 1 could be supplemental. Regarding this figure it is unclear why the distribution of CAF1 in the polyosome profile is so different in panels A and B (control). In principle, these panels should be very similar. I suggest to delete Caf1 if it is not reproducible or to confirm the result.

Authors: See response above.

Referee 1: Figure 3D. Some bars lack error bars.

Authors: Values are expressed relative to the wild-type value in each experiment.
Therefore, the wild-type error bars are not visible. This is explained in the figure legend.

Referee 1: Figure 5C. The data obtained upon amino acid starvation is not convincing. There is hardly any difference for the not4 delta strain. In contrast the differences obtained upon glucose starvation are clear. Therefore I suggest to delete figure 5C, which is not compelling and not necessary.

Authors: See response above.

Referee 1: Page 18, line 470. Translation initiation and decapping are not reciprocal but competing processes.

Authors: We thank the referee for making us aware of this mistake. We say now: "Translation initiation and mRNA decapping are competing processes."

Referee 1: Page 18, lines 479-483. The discussion regarding the Meijer et al. paper should be deleted because the findings reported in this paper could not be reproduced in subsequent studies. First, eIF4A2 does not interact with the CCR4-NOT complex as shown by Chen et al. 2014b, Mathys et al. 2014 and also Rouya et al., 2014. Secondly, eIF4A2 is not recruited but displaced from repressed mRNAs (Fukao et al.2014, Fukaya et al.,2014). If the authors refer to the Meijer et al. paper they should also state that many other papers reached different conclusions, to provide an accurate view of the literature. However, this discussion is not relevant to this paper and therefore it is easier to delete any reference to the Meijer et al. paper. Furthermore mRNA destabilization can occur cotranslationally as shown by Hu et al.(at least in yeast).

Authors: We were not aware of these circumstances. We thank the referee for making this point. We removed the discussion of the findings of Meijer et al. and do not cite this paper anymore.