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

Steffen Preissler[1,2,†,#,*], Julia Reuther[1,2,#], Miriam Koch[1,2,‡], Annika Scior[1,2,§], Michael Bruderek[1,¶], Tancred Frickey[3] & Elke Deuerling[1,**]

Abstract

Translation of aberrant or problematic mRNAs can cause ribosome stalling which leads to the production of truncated or defective proteins. Therefore, cells evolved cotranslational quality control mechanisms that eliminate these transcripts and target arrested nascent polypeptides for proteasomal degradation. Here we show that Not4, which is part of the multifunctional Ccr4 Not complex in yeast, associates with polysomes and contributes to the negative regulation of protein synthesis. Not4 is involved in translational repression of transcripts that cause transient ribosome stalling. The absence of Not4 affected global translational repression upon nutrient withdrawal, enhanced the expression of arrested nascent polypeptides and caused constitutive protein folding stress and aggregation. Similar defects were observed in cells with impaired mRNA decapping protein function and in cells lacking the mRNA decapping activator and translational repressor Dhh1. The results suggest a role for Not4 together with components of the decapping machinery in the regulation of protein expression on the mRNA level and emphasize the importance of translational repression for the maintenance of proteome integrity.

Keywords Ccr4 Not complex; Not4; protein homeostasis; ribosome stalling; translational repression
Subject Categories Protein Biosynthesis & Quality Control; RNA Biology

Introduction

Protein synthesis is controlled on multiple levels to maintain the integrity of the cellular proteome. Therefore, diverse quality control mechanisms evolved to prevent production of defective proteins, which are, for example, encoded by aberrant messenger RNAs (mRNAs) that arise from mutations or errors during transcription and mRNA processing. Translation of aberrant mRNAs commonly causes ribosome stalling, which is recognized by quality control systems that prevent further synthesis of faulty proteins. These systems include mRNA surveillance pathways that cotranslationally induce degradation of mRNAs and recycle stalled ribosomes (Graille & Seraphin, 2012).

The turnover of mRNA commonly involves deadenylation of the 3′ end and subsequent removal of the 5′ cap structure (decapping) to inhibit further translation initiation and allow for degradation by 5′ 3′ exonucleases and the exosome (Weill *et al*, 2012). However, rapid deadenylation independent decapping also plays a role in mRNA surveillance and provides an additional mechanism for trans lational control (Muhlrad & Parker, 1994).

In addition, a ribosome bound protein quality control system was recently discovered that facilitates the degradation of arrested nascent polypeptides (Bengtson & Joazeiro, 2010; Brandman *et al*, 2012). A key component of this system is the E3 ubiquitin protein ligase Ltn1. Ltn1 binds to disassembled 60S ribosomal subunits and ubiquitinates arrested polypeptides which result, for example, from the translation of *non stop* (*NS*) mRNAs that lack an in frame termi nation codon (Bengtson & Joazeiro, 2010). Ribosomes that translate *NS* mRNAs are thought to enter the 3′ poly(A) tail where they become stalled by the synthesis of consecutive lysine residues. Such polybasic sequences likely induce ribosome stalling by electrostatic interactions within the ribosomal exit tunnel (Lu & Deutsch, 2008; Brandman *et al*, 2012; Charneski & Hurst, 2013).

In yeast, another E3 ligase, Not4, was suggested to be involved in cotranslational protein quality control (Dimitrova *et al*, 2009; Matsuda *et al*, 2014). Not4 is part of a large molecular assembly, the Ccr4 Not complex, which consists of at least nine core subunits (Ccr4, Caf1, Caf40, Caf130, Not1 5) (Chen *et al*, 2001). Among them, Not1 is essential for yeast viability and forms the scaffold of the complex (Maillet *et al*, 2000). The complex is evolutionarily

1 Molecular Microbiology, University of Konstanz, Konstanz, Germany
2 Konstanz Research School Chemical Biology, University of Konstanz, Konstanz, Germany
3 Applied Bioinformatics, University of Konstanz, Konstanz, Germany
*Corresponding author. Tel: +44 1223 769100; E mail: sp693@cam.ac.uk
**Corresponding author. Tel: +49 7531 882647; E mail: elke.deuerling@uni konstanz.de
#These authors contributed equally to this work
†Present address: University of Cambridge, Cambridge Institute for Medical Research, Cambridge, UK
‡Present address: Department of Chemistry and Biochemistry, University of Bern, Bern, Switzerland
§Present address: Leibniz Institut für Molekulare Pharmakologie, Campus Berlin Buch, Berlin, Germany
¶Present address: Institute of Biochemistry and Molecular Biology, University of Bonn, Bonn, Germany

conserved in eukaryotes and localizes to the nucleus and cytosol. In the nucleus, the Ccr4 Not complex has been implicated in the regulation of transcription, whereas an important cytosolic function involves the Ccr4 and Caf1 subunits, which constitute the major deadenylases of yeast cells and catalyse poly(A) tail shortening of mRNAs to initiate their degradation (Tucker *et al*, 2001).

Not4 contains an N terminal RING domain required for its ubiquitination activity (Mulder *et al*, 2007) and has been suggested to regulate the levels of the histone demethylase Jhd2, the catalytic subunit of the DNA polymerase α Cdc17, the transcription factor Yap1, the nascent polypeptide associated complex NAC and the ribosomal protein Rps7A (Panasenko *et al*, 2006; Mersman *et al*, 2009; Haworth *et al*, 2010; Gulshan *et al*, 2012; Panasenko & Collart, 2012). In addition, the deletion of *NOT4* affects cellular protein homeostasis (Halter *et al*, 2014), and based on the observation that the levels of cotranslationally arrested polypeptides were increased in cells lacking Not4, it was proposed that Not4 ubiquitinates arrested nascent polypeptides to target them for proteasomal degradation (Dimitrova *et al*, 2009). In contrast, other studies suggest that deletion of *NOT4* enhances ubiquitination of nascent chains and does not affect the degradation of arrested translation products (Bengtson & Joazeiro, 2010; Duttler *et al*, 2013). Given these contradictory results, the function of Not4 in cotranslational quality control remains unclear.

Here we demonstrate that Not4 plays a crucial role in cotranslational quality control; however, it does not contribute to the ubiquitination and turnover of arrested nascent polypeptides. Instead, our data indicate that Not4 is required for global translational repression under nutritional limitations and especially for repression of mRNAs that cause transient ribosome stalling. This function likely involves the decapping components Dhh1 and Dcp1. Thus, Not4 dependent translational repression adds an additional level of cotranslational quality control important for the maintenance of cellular protein homeostasis.

Results

Not4 and its complex partners associate with polysomes

In yeast cells, Not4 has been shown to migrate with polysomes in sucrose gradients (Dimitrova *et al*, 2009), suggesting an interaction with ribosomal particles. To analyse this interaction in more detail, we prepared yeast cell lysates and separated the different ribosomal species by density gradient centrifugation. The gradient fractions were immunoblotted to detect Not4. Rpl25, a protein of the 60S ribosomal subunit, and the ribosome associated chaperone Zuo1, which binds to the 60S subunit, were detected as controls. While Rpl25 and Zuo1 were present in all fractions containing 60S ribosomal subunits, the strongest signals for Not4 were found in late polysomal fractions (Fig 1A).

Caf1, another subunit of the Ccr4 Not complex, was distributed throughout the gradient, but the majority was also detected in late polysomal fractions (Fig 1A). Moreover, HA tagged versions of Ccr4, Not1 and Not5 showed a similar distribution (Supplementary Fig S1A), suggesting that the entire Ccr4 Not complex interacts with polysomes.

To confirm the interaction between the Ccr4 Not complex and polysomes, we treated wild type cell lysate with RNase A prior to loading on sucrose gradients to degrade the mRNA and to convert polysomes into 80S monosomes (Fig 1B). The Not4 and Caf1 signals shifted to the 80S fractions upon RNase A treatment and to the non ribosomal top fractions. The same was observed for Zuo1 (Supplementary Fig S1B). 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 (Fig 1C).

Not4 inhibits the expression of polylysine-arrested proteins

Polysomes consist mainly of translating ribosomes but can also contain large jammed assemblies that result from stalling events when ribosomes encounter obstacles during their migration along mRNAs. As Not4 interacted predominantly with very large polysomes, we hypothesized that it might be recruited to stalled ribosomes. Ribosome stalling leads to subunit disassembly followed by Ltn1 mediated ubiquitination of the nascent chains and their proteasomal degradation (Bengtson & Joazeiro, 2010; Brandman *et al*, 2012; Shao & Hegde, 2014). To investigate whether Not4 plays a role during cotranslational quality control, we analysed the expression of arrested polypeptides in the presence and absence of Not4 and Ltn1 using reporter proteins that transiently stall ribosomes during translation. These reporters consisted of an N terminal GFP moiety fused to a Flag tag and the His3 protein (Fig 2A). To induce ribosome stalling, twelve consecutive lysine residues (K12) were either inserted between GFP and the Flag tag (GFP K12 Flag His3; called hereafter K12 M) or fused to the C terminal end (GFP Flag His3 K12; called hereafter K12 C). The same protein without a lysine stretch (K0) served as a non arrested reporter. The levels of the K0 and K12 polypeptides in wild type and mutant cells were then analysed by immunoblotting with antibodies directed against GFP and the Flag tag to detect arrest products and full length proteins.

While the K0 reporter was produced in all strains at similar levels, no or only weak signals for both K12 arrest products were detected in wild type and *not4*Δ cells, indicating that the arrested nascent chains were efficiently degraded (Fig 2B, C and quantified in D). As observed earlier (Bengtson & Joazeiro, 2010), the levels of K12 arrested proteins were increased in cells lacking Ltn1. Strikingly, the signals of the arrest products were strongly enhanced up to the level of non arrested K0 proteins when Not4 and Ltn1 were both absent (*not4*Δ*ltn1*Δ) (Fig 2B D). Moreover, most stalled K12 M polypeptides detectable in *ltn1*Δ and *not4*Δ*ltn1*Δ cells were still bound to ribosomes although a significant portion of K12 M peptides was released into the supernatant in *not4*Δ*ltn1*Δ cells (Supplementary Fig S2).

The polylysine stretches of released K12 M and K12 C arrest products may provide exposed ubiquitination sites that could unequally influence their posttranslational stability in the different knockout strains. We could rule out this possibility since polyproline induced ribosome stalling (Gutierrez *et al*, 2013) using a P12 M construct (GFP P12 Flag His3) similarly increased arrest product levels in the *ltn1*Δ and *ltn1*Δ*not4*Δ mutants, even though the arrest was much weaker compared to the K12 M construct as evident by efficient production of full length protein in all strains (Fig 2E).

Taken together, the data show that the loss of Not4 in addition to Ltn1 enhanced the expression of arrested nascent polypeptides and increased ribosome stalling and subsequent release of truncated

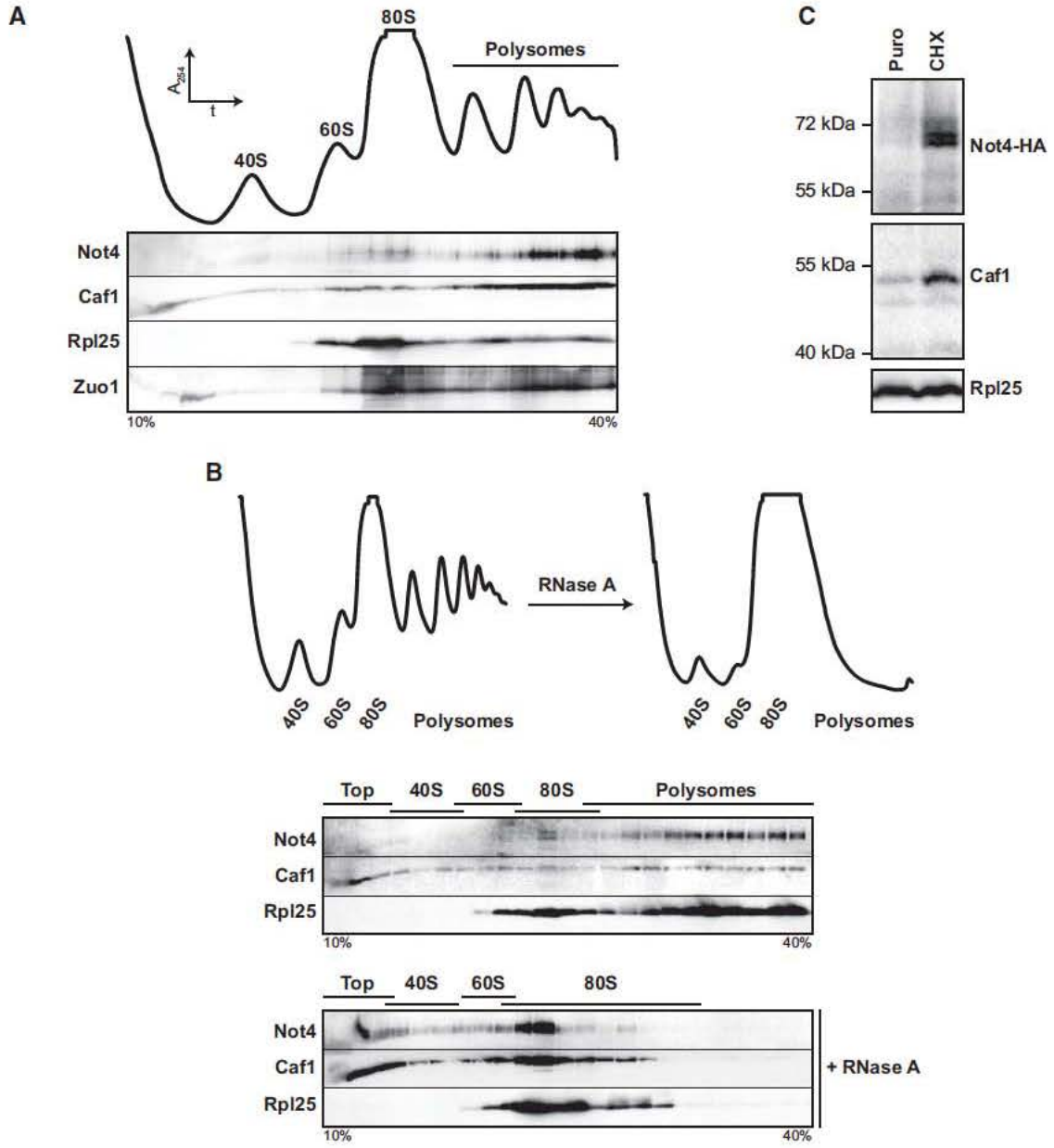

Figure 1. Not4 interacts with polysomes.

A Ribosomes from wild type (wt) yeast lysates were separated on a 10–40% sucrose gradient. Top: Absorbance profile at 254 nm (A_{254}). Bottom: Protein fractions were analysed by Western blotting using antibodies directed against the proteins indicated.

B Ribosomal particles from an RNase A treated and untreated control lysate were separated by density gradient centrifugation. Top: A_{254} profiles. Bottom: Western blot analysis.

C *not4*Δ cells expressing HA tagged Not4 (Not4 HA) from a plasmid were grown to an optical density (OD_{600}) of 0.8. A lysate was prepared, and one half was treated with puromycin (Puro) to release nascent polypeptides and mRNA, while the other half was treated with cycloheximide (CHX) to stall translation. Samples were layered on top of a 20% sucrose cushion, and ribosomes were sedimented by ultracentrifugation. Ribosomal pellets were resuspended, and equal amounts of ribosomes were applied to Western blot analysis. Not4 HA was detected with antibodies directed against the HA epitope tag. Rpl25 was detected as a loading control.

Source data are available online for this figure.

polypeptides. Thus, defective polypeptides can escape cotranslational quality control in the absence of Not4 and Ltn1 and accumulate in the cytosol.

Since ribosome stalling on K12 or P12 sequences is transient, we analysed whether Not4 also inhibits the expression of arrested polypeptides when ribosomes encounter insurmountable obstacles. Translation of *non stop* (*NS*) mRNAs, which lack an in frame stop codon, likely proceeds into the 3′ untranslated region (UTR) and the poly(A) tail where ribosomes become stalled by the synthesis of long polylysine sequences or on the 3′ end of the mRNA, leading to

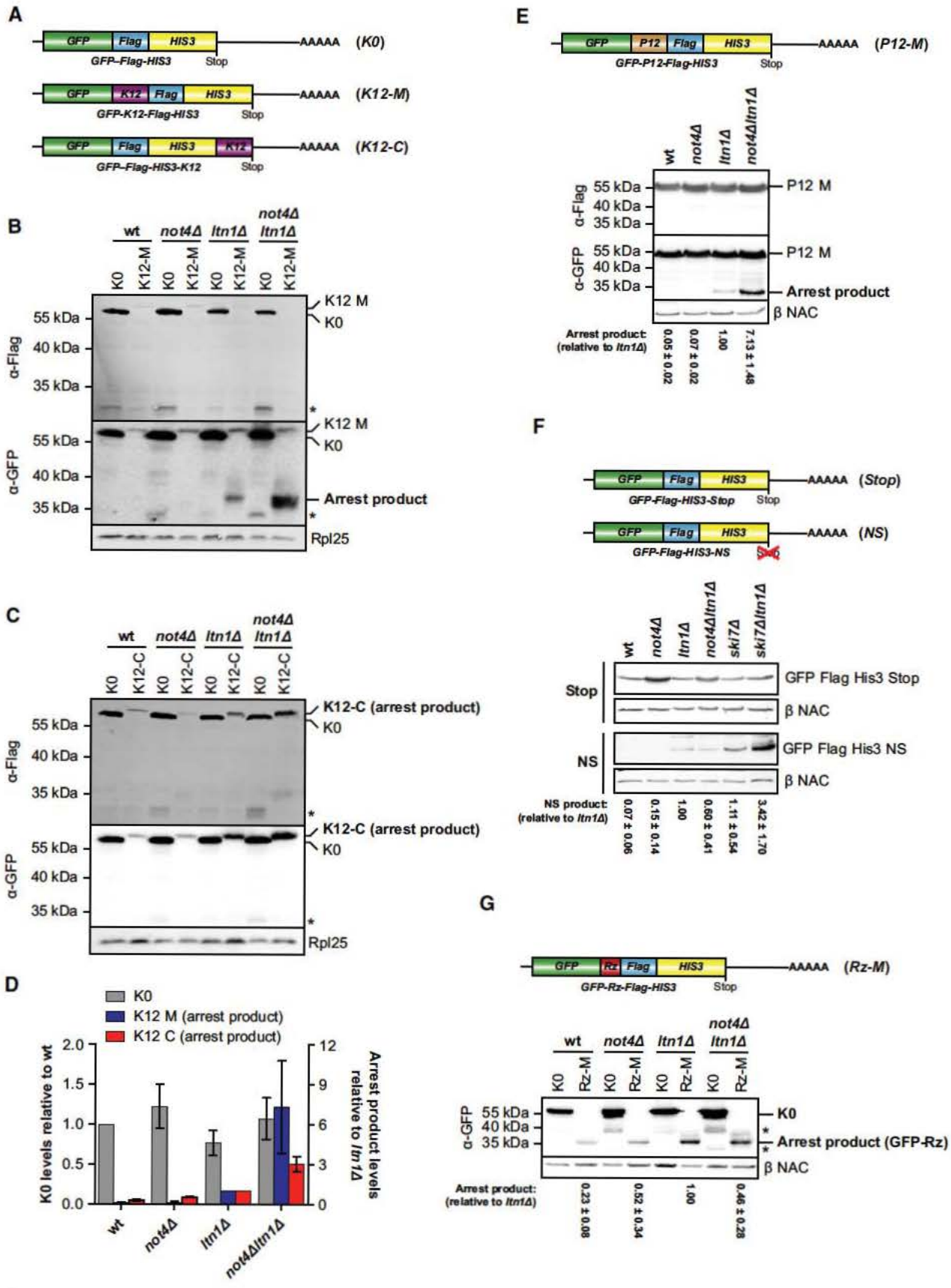
A
GFP
Flag
HIS3
AAAAA
(K0)
GFP–Flag-HIS3
Stop
K12
(K12-M)
GFP-K12-Flag-HIS3
(K12-C)
GFP–Flag-HIS3-K12
B
wt
not4Δ
ltn1Δ
not4Δ ltn1Δ
K0
K12-M
α-Flag
α-GFP
55 kDa
40 kDa
35 kDa
K12 M
Arrest product
Rpl25
C
K12-C
K12-C (arrest product)
D
K0
K12 M (arrest product)
K12 C (arrest product)
K0 levels relative to wt
Arrest product levels relative to ltn1Δ
E
P12
(P12-M)
GFP-P12-Flag-HIS3
P12 M
β NAC
Arrest product:
(relative to ltn1Δ)
0.05 ± 0.02
0.07 ± 0.02
1.00
7.13 ± 1.48
F
(Stop)
GFP-Flag-HIS3-Stop
(NS)
GFP-Flag-HIS3-NS
ski7Δ
ski7Δltn1Δ
Stop
NS
GFP Flag His3 Stop
GFP Flag His3 NS
NS product:
(relative to ltn1Δ)
0.07 ± 0.06
0.15 ± 0.14
1.00
0.60 ± 0.41
1.11 ± 0.54
3.42 ± 1.70
G
Rz
(Rz-M)
GFP-Rz-Flag-HIS3
Rz-M
Arrest product (GFP-Rz)
Arrest product:
(relative to ltn1Δ)
0.23 ± 0.08
0.52 ± 0.34
1.00
0.46 ± 0.28

Figure 2.

Figure 2. Not4 inhibits expression of polybasic translation arrest products.

A Schematic of mRNA encoding the non stalling GFP Flag His3 (K0) control construct or ribosome stalling constructs where twelve consecutive lysine residues were inserted between GFP and Flag (GFP K12 Flag His3; K12 M) or fused to His3 (GFP Flag His3 K12; K12 C).

B, C Yeast cells transformed with centromeric plasmids expressing either K0 construct or K12 M (B) or K12 C ribosome stalling construct (C) were grown in SCD His to an optical density (OD_{600}) of 0.8, and normalized lysates were analysed by Western blotting. Full length proteins and translation arrest products were detected with GFP specific (α GFP) and Flag specific (α Flag) antibodies. Rpl25 was detected as a loading control. The asterisk marks degradation products.

D Quantification of full length K0 levels (n = 6, plotted on the left y axis) as well as K12 M (n = 6) and K12 C (n = 3) arrest product levels (plotted on the right y axis) from independent experiments as shown in (B) and (C). The values were normalized to the loading control, and arrest product levels are expressed relative to *ltn1*Δ cells (set to 1). Mean ± SD bars are shown.

E Top: Schematic of mRNA encoding the P12 M polyproline ribosome stalling construct GFP P12 Flag His3. Bottom: Same experiment as in (B) performed with P12 M and β NAC was detected as a loading control. Arrest product levels were quantified as in (D). Shown is mean ± SD (n = 3).

F Top: Schematic of mRNA with a *HIS3 3′* untranslated region as described in Ito Harashima *et al* (2007) encoding GFP Flag His3 fusion protein (Stop) or non stop (NS) protein. Bottom: The experiment was performed as in (B) with Stop and NS constructs. β NAC was detected as a loading control. Arrest product levels were quantified as in (D). Shown is mean ± SD (n = 5).

G Top: Schematic of the *Rz M* mRNA containing a self cleavable hammerhead ribozyme sequence (Rz; red) inserted into the open reading frame. Bottom: The experiment was performed as in (B) with the Rz M construct. β NAC served as a loading control. Arrest product levels were quantified as in (D). Shown is mean ± SD (n = 3). Asterisks mark a degradation product of K0.

Source data are available online for this figure.

destruction of the transcript (Inada & Aiba, 2005) and degradation of the nascent chain (Ito Harashima *et al*, 2007). According to previous observations (Bengtson & Joazeiro, 2010), the level of NS proteins was enhanced in *ltn1*Δ mutants (Fig 2F). However, the combined deletion of *NOT4* and *LTN1* did not further increase NS protein levels. As a positive control, we included cells lacking Ski7, a protein that plays a role in NS mRNA surveillance (van Hoof *et al*, 2002) and inhibits NS protein expression (Bengtson & Joazeiro, 2010). Deletion of *SKI7* indeed increased NS protein levels, and this effect was stronger in *ski7*Δ*ltn1*Δ mutants (Fig 2F).

We also analysed ribosome stalling at the end of truncated mRNAs by introducing a self cleaving RNA segment, the hammerhead ribozyme (Rz), between the GFP and Flag encoding sequence to obtain the GFP Rz Flag His3 fusion construct (Rz M, Fig 2G). The Rz sequence cuts the mRNA site specifically *in cis* after transcription, which generates truncated mRNAs that cause ribosome stalling at the cleavage site (Tsuboi *et al*, 2012). Thus, the translation arrest product (GFP Rz) of the construct can be detected by GFP specific antibodies. Expression of GFP Rz was weak in wild type and *not4*Δ cells and increased in cells lacking Ltn1 (Fig 2G). Simultaneous deletion of *NOT4* and *LTN1* did not further increase the level of arrested polypeptides (the level was rather decreased relative to *ltn1*Δ).

We conclude that whereas in general Ltn1 is required to prevent the accumulation of translation arrest products, Not4 acts more specifically and inhibits the expression of transiently arrested proteins, but not of those that result from ribosome stalling on *NS* or truncated mRNAs. Translation arrest on *NS* or truncated mRNAs is likely stronger, and the topology of stalled ribosomes on the 3′ end of an mRNA is different and thus may require other quality control mechanisms.

Not4 functions in translational repression

Not4 could inhibit the expression of transiently arrested polypeptides by different mechanisms including: (i) destabilization of arrest products, (ii) translational repression or (iii) enhanced turnover of mRNAs that cause ribosome stalling.

The observation that deletion of *NOT4* alone does not increase arrest product levels challenges the hypothesis that Not4 contributes directly to the degradation of arrested polypeptides. Accordingly, the stability of K12 M and P12 M arrest products was similar in *ltn1*Δ and *not4*Δ*ltn1*Δ cells (Supplementary Fig S3) and thus does not explain the strong increase of arrest product levels in *not4*Δ*ltn1*Δ mutants.

An alternative scenario could be that Not4 contributes to translational repression, which restricts arrest product synthesis. To investigate this possibility, we analysed the effect of Not4 on K12 reporter synthesis independent from degradation of the arrested products. We thus generated constructs which contain the 2A sequence of FMDV (foot and mouth disease virus) (Fig 3A). Insertion of 2A between GFP and Flag induces polylysine independent ribosome pausing at the end of the 2A encoding sequence and rapid release of a GFP 2A fragment from a subset of nascent chains, followed by translation "re initiation" and synthesis of the downstream products by the same ribosomes (Donnelly *et al*, 2001; Doronina *et al*, 2008). Therefore, the GFP 2A levels report on translation efficiency regardless of the stability of the full length protein (Ito Harashima *et al*, 2007). As the 2A arrested product is cotranslationally released, it should escape destabilization by Ltn1 and thus reveal the effect of Not4 on reporter translation.

We analysed the expression of GFP 2A Flag His3 K12 (2A K12) containing a K12 stalling sequence at the C terminus, and GFP 2A Flag His3 (2A K0), which lacks a C terminal stalling sequence (Fig 3A). The mRNA levels of both constructs were similar in all strains, and only the *2A K12* mRNA levels were slightly elevated in *not4*Δ cells (Fig 3B). In agreement with the data shown above, arrested full length 2A K12 protein could only be detected in *ltn1*Δ cells (Fig 3C) and the signal was further enhanced in *not4*Δ*ltn1*Δ mutants, whereas full length 2A K0 was expressed in all strains (Fig 3D). Importantly, the N terminal GFP 2A fragment of 2A K12 and 2A K0 was produced in all strains independent of *LTN1* deletion albeit detectable only at lower levels (Fig 3C). Nevertheless, the GFP 2A levels of the 2A K12 and 2A K0 reporter constructs were significantly elevated (~2 to 3 fold) in *not4*Δ and *not4*Δ*ltn1*Δ mutants (Fig 3C and D). A similar tendency was observed for full length 2A K0, whereas the levels of the K0 construct which lacks the 2A element were less increased in these strains (compare Figs 3D and 2D). Thus, loss of Not4 enhanced the synthesis of 2A containing proteins independent of Ltn1. Together, these data are consistent with a role of Not4 in translational repression induced by transient ribosome stalling within open reading frames.

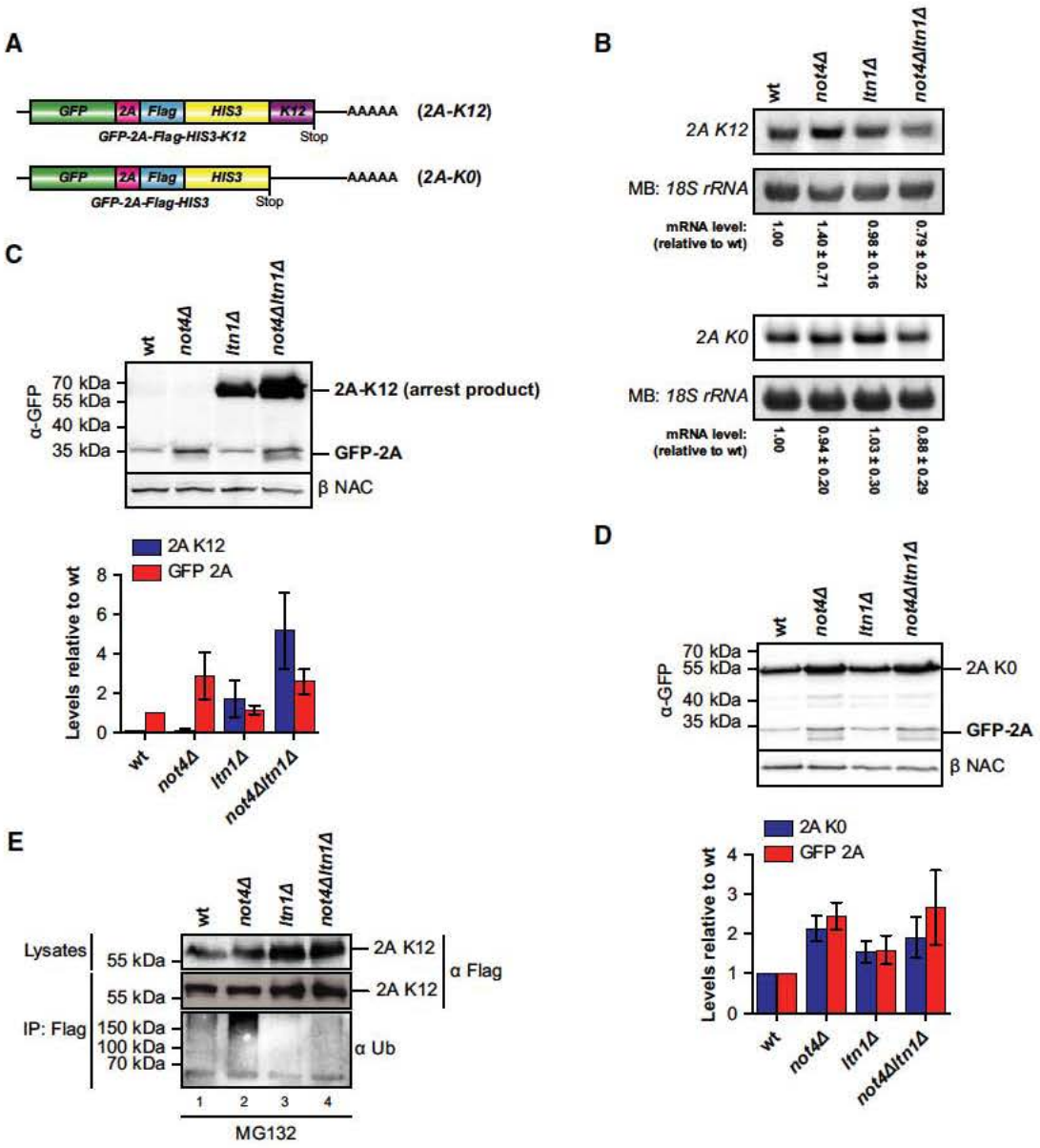

Figure 3. Not4 acts in translational repression.

A Schematic of mRNA encoding the reporter constructs GFP 2A Flag His3 (2A K0) and GFP 2A Flag His3 K12 (2A K12) containing an in frame insertion of the FMDV 2A sequence.

B Northern blot analysis of *2A K12* and *2A K0* mRNA levels in yeast cells. The membrane was stained with methylene blue (MB) to visualize the 18S ribosomal RNA (rRNA) as a loading control. The reporter mRNA signals were quantified and normalized to the loading control. Shown is mean ± SD (n = 4 for *2A K12* and n = 5 for *2A K0*).

C, D Lysates of yeast cells expressing 2A K12 (C) or 2A K0 (D) were analysed by Western blotting with antibodies against GFP (α GFP) and β NAC. Bar graph: Western blot signals of full length proteins and GFP 2A of three independent experiments were quantified, normalized to the loading control and expressed relative to values in wild type (wt) cells. Shown is mean ± SD (n = 3).

E Yeast cells were transformed with a plasmid expressing the ribosome stalling construct 2A K12. Cells were grown in SCD His medium to the mid log phase and treated with MG132. Lysates were prepared and the fusion proteins were immunoprecipitated. Samples of the lysates and the precipitated proteins were analysed by Western blotting. Proteins were detected with Flag specific antibodies, and ubiquitination was detected with ubiquitin specific (α Ub) antibodies. Similar results were obtained in at least two separate experiments.

Source data are available online for this figure.

Our results furthermore disfavour a function of Not4 in degrada tion of arrested polypeptides. To investigate this directly, we ana lysed ubiquitination of 2A K12 proteins. Cells were treated with the proteasome inhibitor MG132 prior to lysis to prevent degradation of arrested nascent chains, and therefore, full length 2A K12 was detected in wild type and *not4*Δ cells (Fig 3E). 2A K12 proteins were

then immunoprecipitated and analysed for ubiquitination. 2A K12 ubiquitination was detected in wild type cells but not in *ltn1*Δ mutants (Fig 3E; Bengtson & Joazeiro, 2010; Brandman *et al*, 2012). In contrast, 2A K12 ubiquitination was strongly enhanced in *not4*Δ cells, which is consistent with increased reporter synthesis in the absence of Not4 but argues further against a role of Not4 in ubiquitination of arrested polypeptides. The simultaneous deletion of *LTN1* and *NOT4* reduced ubiquitination of 2A K12 proteins back to the level of *ltn1*Δ cells. This again implies that Ltn1 ubiquitinates arrested K12 proteins, whereas Not4 does not. In addition, expression of the E3 ligase deficient mutant Not4 L35A (Mulder *et al*, 2007) efficiently reduced arrest product levels in *not4*Δ*ltn1*Δ cells to the *ltn1*Δ level (Supplementary Fig S4), suggesting that Not4 mediated inhibition of arrest product expression does not require its E3 ligase activity.

Altered mRNA levels have a minor effect on the expression of polylysine-arrested polypeptides

We observed increased expression and ubiquitination of K12 arrested polypeptides in the absence of Not4 and assumed that this is due to a loss of translational repression. However, it has been shown earlier that mutations that interfere with mRNA decay enhance protein expression and accordingly cotranslational ubiquitination (Duttler *et al*, 2013). We therefore addressed whether Not4 influences steady state mRNA levels of the K12 M reporter since the arrest products of this construct were strongly enhanced in *not4*Δ*ltn1*Δ cells (Fig 2B and D). Indeed, the levels of the *K12 M* mRNA were elevated about twofold in the absence of Not4, while the levels of the non stalling *K0* mRNA were similar in all strains (Fig 4A). Thus, increased mRNA levels may contribute to enhanced K12 M protein expression in *not4*Δ*ltn1*Δ cells and hence to increased ribosome stalling and nascent chain ubiquitination. The mRNA half lives of both, the *K0* and *K12 M* mRNAs, were moderately elevated in the absence of Not4, which may explain the increased levels of the *K12 M* mRNA in *not4*Δ cells (Fig 4A; Supplementary Fig S5). 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.

Since Not4 is part of the Ccr4 Not complex, we analysed reporter mRNA levels also in other *ccr4 not* mutants, including cells lacking the major mRNA deadenylases Ccr4 and Caf1 (Fig 4B). We found that the mRNA levels of K12 M were similarly increased in *not4*Δ mutants and in cells lacking Ccr4 and Caf1. However, in contrast to *not4*Δ cells, also the *K0* mRNA signals were increased in *ccr4*Δ and *caf1*Δ mutants, respectively. This agrees well with a general role of Ccr4 and Caf1 in mRNA decay and suggests a more specific effect of Not4 on the stability of ribosome stalling mRNAs.

We then directly compared the effects of *NOT4* or *CCR4* deletion on *K12 M* mRNA levels and the levels of the corresponding translation arrest product in the absence of Ltn1. *K12 M* mRNA levels were similarly increased in *ccr4*Δ*ltn1*Δ and *not4*Δ*ltn1*Δ cells, whereas the arrest product levels were only moderately enhanced in *ccr4*Δ*ltn1*Δ mutants compared to *ltn1*Δ cells (Fig 4C). Only the combined deletion of *LTN1* and *NOT4* resulted in a strong increase of arrest product levels. Thus, although *K12* mRNA levels influence reporter expression, they do not account for the strongly elevated K12 protein levels in *not4*Δ*ltn1*Δ mutants. This suggests that the effect of Not4 on the expression levels of K12 arrested polypeptides is mainly caused by translational repression.

Not4 and decapping proteins are required for fast global translational repression upon nutrient withdrawal

To further investigate the potential role of Not4 in translational repression, we took advantage of earlier observations that cells repress overall translation in response to a variety of stresses to prevent accumulation of defective proteins. It is known that yeast cells lacking the mRNA decapping proteins Dcp1 and Dcp2 as well as the decapping activator Dhh1 show defects in fast translational repression upon nutrient withdrawal (Holmes *et al*, 2004; Coller & Parker, 2005), a condition that rapidly reduces the cellular concentration of aminoacyl tRNAs and may promote ribosome stalling. Interestingly, a physical interaction between the Ccr4 Not complex and Dhh1 has been reported in yeast (Hata *et al*, 1998; Coller *et al*, 2001; Maillet & Collart, 2002; Rouya *et al*, 2014). Therefore, we investigated whether loss of Not4 or Dhh1 causes a defect in translational repression after nutrient withdrawal.

Glucose depletion caused the rapid conversion of polysomes into 80S monosomes in wild type cells, reflecting severe reduction of translation activity (Fig 5A). In contrast, residual polysome peaks were still detected in *dhh1*Δ mutants after glucose withdrawal, which agrees well with the reported defect in translational repression. Importantly, cells lacking Not4 showed a similar defect (Fig 5A) and the relative rate of protein synthesis upon glucose depletion was higher in *not4*Δ mutants than in wild type cells (Fig 5B). Translational repression in *dhh1*Δ and *not4*Δ cells was also affected shortly after amino acid withdrawal as evident by the smaller decrease of polysomes (Fig 5C and D). These data suggest that Dhh1 and Not4 are both important for fast translational repression during nutrient starvation.

Not4 and decapping proteins are required for translational repression during ribosome stalling

Based on the similar defects of cells lacking Not4 or Dhh1 in global translational repression upon nutrient withdrawal, we investigated whether loss of Dhh1 and Dcp1 Dcp2 decapping complex function might also increase expression of translation arrest products at normal growth conditions. As we were unable to delete *DCP1* or *DCP2* in our yeast strain, we introduced a genomic point mutation in *DCP1* (*dcp1 34*), which causes strong loss of function (Tharun & Parker, 1999). Growth of *dhh1*Δ cells was only slightly impaired at 30°C, whereas *not4*Δ and *dcp1 34* mutants had a pronounced growth defect (Fig 6A). Insertion of a *kanMX* cassette at the *DCP1* locus, which was required for *dcp1 34* construction, did not affect growth. Further mutational analysis revealed that *not4*Δ*dhh1*Δ double mutants were viable in our strain background and showed only a slightly increased growth defect (Fig 6A).

To investigate translation arrest product levels in the decapping mutants, we additionally deleted *LTN1*, which did not significantly influence the growth defects (Fig 6A). Whereas no K12 M arrest products were detected in *dhh1*Δ cells, the combined deletion of *DHH1* and *LTN1* increased the level of K12 M arrest products compared to *ltn1*Δ cells, but not the level of the non arrested K0

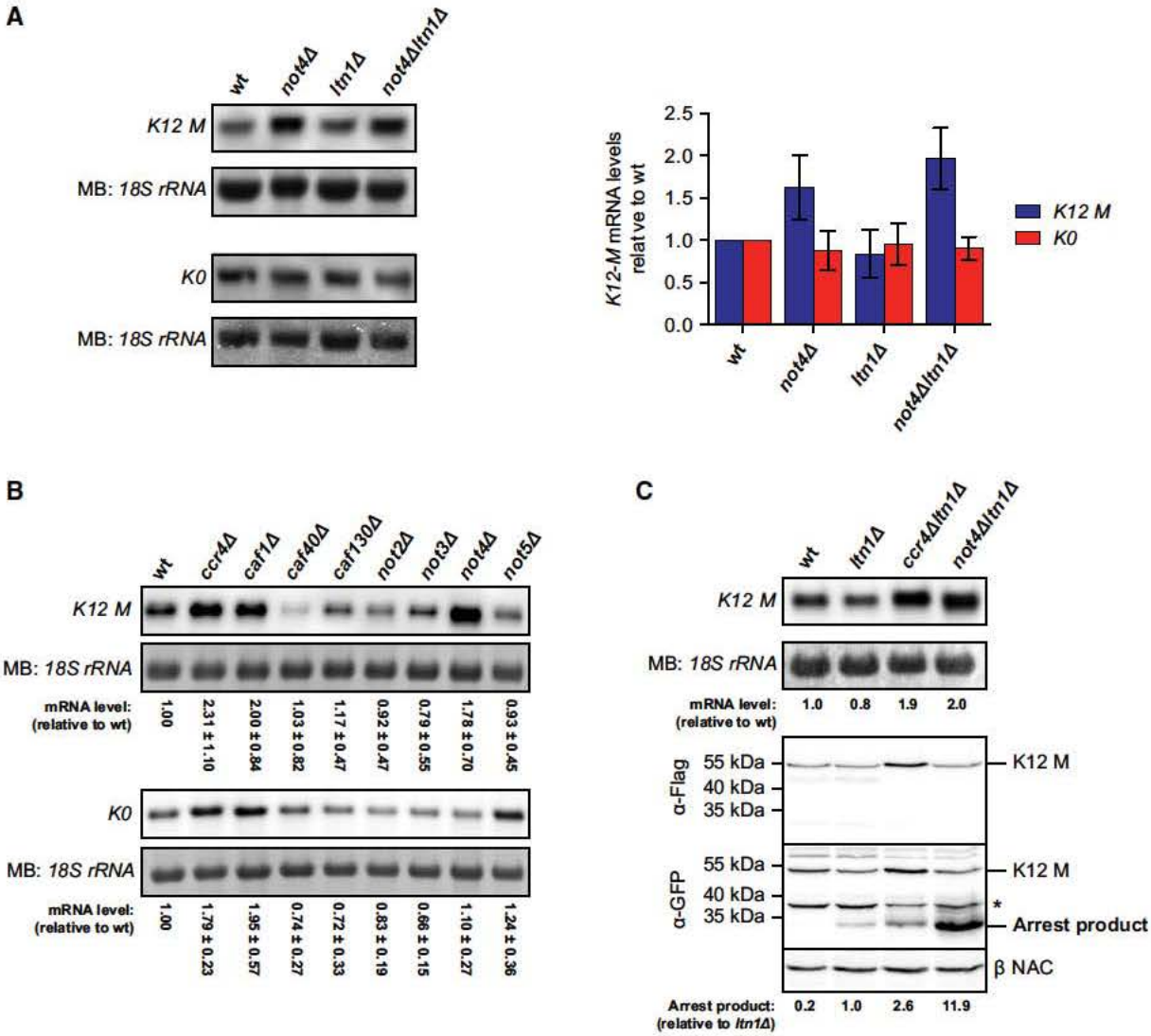

Figure 4. Altered mRNA levels have a minor influence on expression of arrested proteins.

A Northern blot analysis of *K12 M* or *K0* mRNA levels in yeast cells. The membrane was stained with methylene blue (MB) to detect the 18S ribosomal RNA (rRNA) as a loading control. Bar graph: The mRNA signals were quantified, normalized to the loading control and expressed relative to wild type (wt). Shown is mean ± SD (n = 4 for *K12 M* and n = 3 for *K0*).

B Northern blot analysis as in (A) of *K12 M* and *K0* mRNA levels in *ccr4 not* mutants. Shown is mean ± SD (n = 4 for *K12 M* and n = 3 for *K0*).

C Parallel analysis of *K12 M* mRNA levels (top) and K12 M protein levels (bottom). Northern blot analysis was performed as in (A). GFP (α GFP) and Flag specific (α Flag) antibodies were used to detect reporter proteins by Western blotting. Arrest product levels were normalized to the β NAC control signals. The asterisk marks non specific bands. Similar results were obtained in three separate experiments.

Source data are available online for this figure.

proteins (Fig 6B). Moreover, deletion of *LTN1*, *NOT4* and *DHH1* altogether did not further increase the K12 M arrest product level relative to *not4Δltn1Δ* mutants (Fig 6C), suggesting that Dhh1 and Not4 act in the same pathway of translational repression. As antici pated, the K12 M arrest product level was also increased in *ltn1Δdcp1 34* cells, whereas no arrest products were detected in *dcp1 34* single mutants (Fig 6D). The *K12 M* mRNA levels were only increased in *ltn1Δdcp1 34* cells (~2 fold), but not in *dhh1Δltn1Δ* mutants (Fig 6E). Thus, Dhh1 and decapping proteins contribute to inhibition of the synthesis of polybasic proteins. The strong correlation in function and the reported physical association of Dhh1 with the Ccr4 Not complex suggest that the decapping factors Dhh1 and Dcp1 operate together with Not4 in the same pathway. This agrees also with the observed dynamic interaction of Dhh1 with polysomes (Sweet *et al*, 2012). Accordingly, we found HA tagged Dhh1 (Dhh1 HA) comigrating with polysomes in sucrose gradients (Supplementary Fig S6). Dhh1 HA associated with poly somes also in *not4Δ* cells but the signals appeared weaker, suggest ing that Not4 may influence the association of Dhh1 with polysomes. Taken together, these results point to a role of Not4 together with decapping proteins in global and ribosome stalling induced translational repression.

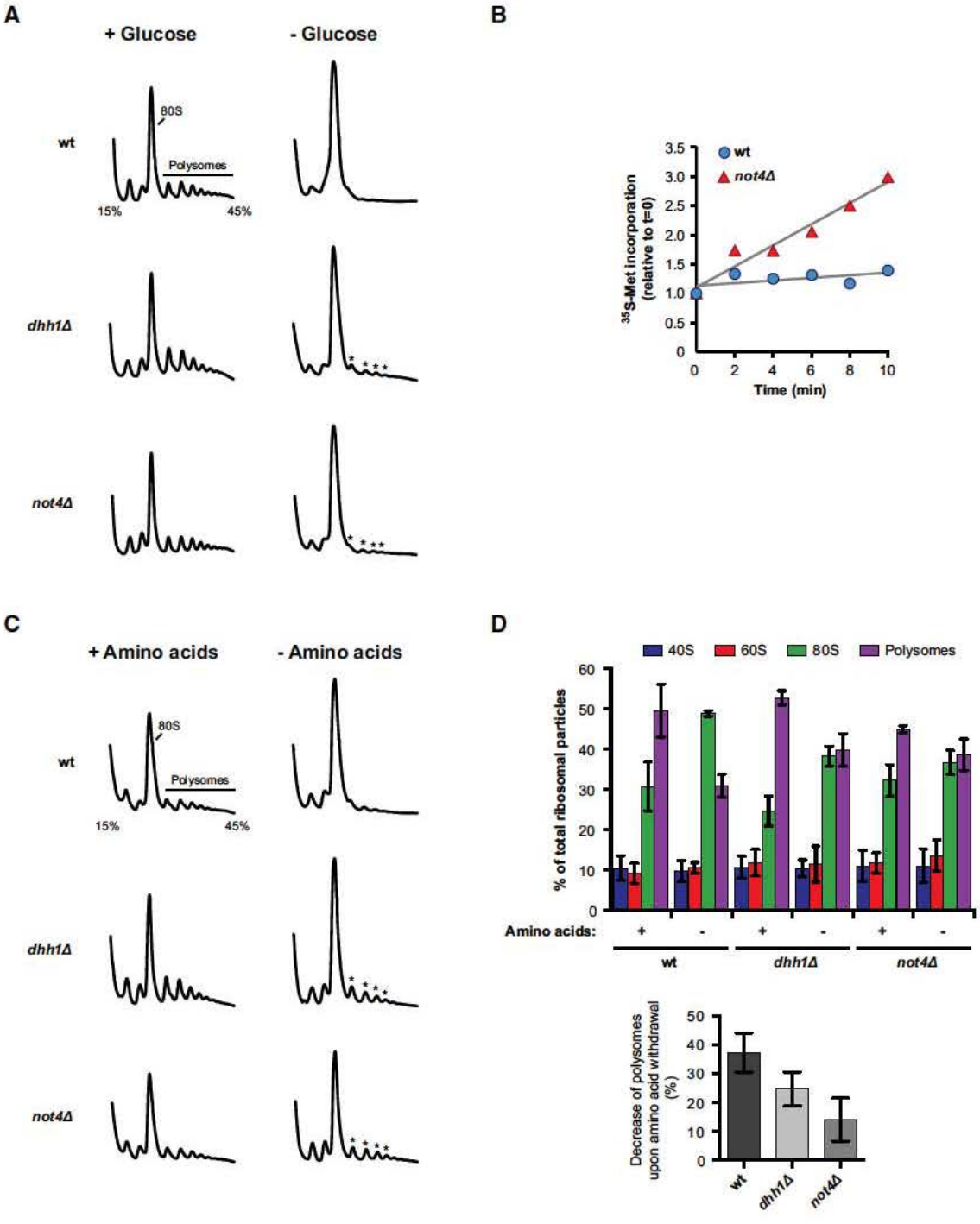

Figure 5. Not4 is required for fast translational repression in response to nutrient withdrawal.

A Polysome profiling with wild type (wt) or mutant yeast cells. Absorbance traces at 254 nm (A_{254}) are shown. Cells were grown to an optical density (OD_{600}) of 0.5 in YPD, pelleted, resuspended in YP with or without 2% glucose and incubated for 10 min. Translation was stopped by the addition of cycloheximide, and cells were collected for polysome profiling on 15–45% sucrose gradients.

B ^{35}S methionine incorporation into proteins after glucose depletion. Cells were grown in SCD medium to OD_{600} 0.5 and transferred to SC labelling medium without glucose containing radioactive ^{35}S methionine. Cells were incubated for 10 min and samples were taken. TCA precipitable radioactivity was measured by liquid scintillation counting. Translation activity is given as incorporated radioactivity relative to $t = 0$. Best fit trendlines are shown in grey.

C, D Polysome profiling of wt and mutant cells as in (A). Cells were grown in SCD medium to OD_{600} 0.5 and transferred to SCD or yeast nitrogen base (YNB) containing 2% glucose without amino acids. Cells were incubated for 10 min prior to polysome analysis. Quantitative analysis of individual ribosome species is shown in (D) with mean values ± SD ($n = 3$).

Source data are available online for this figure.

Not4 and decapping factors are required for the maintenance of cellular protein homeostasis

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 thus addressed whether deregulated translation in *not4*Δ cells interferes with protein homeostasis. Indeed, cells lacking Not4 were unable to grow at elevated temperature (Fig 7A) and expression of the stress inducible chaperone Hsp104 was enhanced in *not4*Δ cells at 30°C (Fig 7B). Induction of the protein stress response was confirmed with reporter constructs consisting of stress responsive promoters of three different genes (*HSP12*, *RPN4* and *HSP104*) fused to a GFP Flag encoding sequence (Fig 7C), indicating constitutive folding stress in *not4*Δ cells. Moreover, we detected severe aggregation of proteins distributed over a broad molecular weight range in *not4*Δ cells at 30°C and aggregates were enriched in proteins larger than 30 kDa (Fig 7D). As a control, we included the analysis of cells lacking the chaperones Ssb1/Ssb2 or Sse1 where predominantly small ribosomal proteins or larger sized proteins aggregate, respectively [Fig 7D and (Koplin *et al*, 2010)]. Mass spectrometry analysis identified more than 500 proteins in the insoluble fraction of *not4*Δ mutants (Supplementary Table S1) including some molecular chaperones such as Hsp104, Ssa1, Sse1 and Ssb1/2, which was confirmed by immunoblotting (Fig 7D). It is difficult to distinguish between aggregated proteins that are directly affected by the absence of Not4 and those that are affected indirectly, for example due to the loss of a binding partner. Nevertheless, sequence analysis of the aggregation prone protein species revealed no obvious common characteristics, such as enhanced hydrophobicity or enrichment of low complexity regions, compared to the non aggregated yeast proteins. However, the mean protein length of aggregated proteins was increased (621 aa for aggregated proteins vs. 412 aa for non aggregated proteins; Supplementary Fig S7A), which is consistent with the enrichment of larger proteins in the insoluble fraction of *not4*Δ cells (Fig 7D). In addition, sequence comparison with genome wide 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; Supplementary Fig S7B). This may reflect high translation rates since there was no obvious correlation between length of the mRNAs and the number of ribosomes associated with them (Supplementary Fig S7C).

Much less insoluble proteins were isolated from *not4*Δ cells grown at 22°C compared to 30°C (Fig 7E). To visualize aggregation *in vivo*, we fused a fluorescent Flag mCherry moiety to enolase 2 (Eno2 Flag mCherry), which was identified in the insoluble fraction of *NOT4* deficient cells (Supplementary Table S1). The fusion protein formed multiple foci in *not4*Δ cells at 30°C but was homogenously distributed at 22°C (Fig 7F). Thus, folding stress induced aggregation can be ameliorated in *not4*Δ cells by reducing the growth temperature.

When cells were pre grown at 22°C, where most proteins were soluble, and then shifted to 30°C to induce aggregation, the simultaneous addition of the translational inhibitor cycloheximide efficiently prevented protein aggregation, while insoluble proteins accumulated in cells without translational inhibition (Fig 7G). This suggests that ongoing protein synthesis is causative for protein aggregation at 30°C in *not4*Δ cells. Similar results were obtained when protein synthesis was reduced by leucine depletion (Fig 7H). Importantly, the turnover of newly made proteins was not significantly impaired in *not4*Δ cells (Supplementary Fig S8A) and *LTN1* deletion had no influence on the accumulation of insoluble polypeptides (Supplementary Fig S8B), indicating that aggregation was not due to defects in cotranslational protein degradation. In addition, aggregation was not increased in cells lacking proteins involved in mRNA degradation such as Ccr4 and Caf1 or in the absence of the 5′ 3′ exonuclease Xrn1 (Supplementary Fig S8C). Moreover, although almost no proteins aggregated in *not4*Δ cells at 22°C, inhibition of K12 M translation arrest product expression was still affected in *ltn1*Δ and *not4*Δ*ltn1*Δ mutants at 22°C (Fig 7I). These data point to a strong correlation between misregulated protein synthesis and aggregation in the absence of Not4 and suggest that loss of Not4 dependent translational control causes severe protein folding stress.

Finally, we hypothesized that if Not4 cooperates functionally with Dhh1 and Dcp1 Dcp2 during translational repression, the proteome integrity should be similarly disturbed in *dhh1*Δ and *dcp1 34* cells. Indeed, strong protein aggregation was detected in *dhh1*Δ and *dcp1 34* mutants and the pattern of insoluble proteins was very similar to *NOT4* deficient cells (Fig 8A). In addition, like in *not4*Δ cells, the Hsp104 chaperone levels were increased in *dhh1*Δ and *dcp1 34* mutants (Fig 8B), indicating constitutive folding stress. Thus, mutations that interfere with translational repression severely affect protein homeostasis.

Discussion

Although several key players of cotranslational quality control in eukaryotes have been identified recently, many details about their

Figure 6. Not4 and Dhh1 act in transcript specific translational repression.

A Spot assay to monitor growth defects of mutant yeast cells. Cells were adjusted to an optical density (OD_{600}) of 0.5, and 5 fold serial dilutions were spotted onto YPD plates. The plates were incubated as indicated.

B D The ribosome stalling K12 M or the non stalling K0 control construct was expressed in wild type (wt) and mutant yeast cells. Normalized lysates were applied to Western blot analysis. Full length proteins and translation arrest products were detected with GFP specific (α GFP) antibodies. β NAC was detected as a loading control. The asterisk indicates unspecific bands. Bar graph: Arrest product levels of three experiments were quantified, normalized to the loading control and expressed relative to *ltn1*Δ. Shown is mean ± SD (n = 3 in B; n = 6 in C and D).

E Northern blot analysis of *K12 M* levels. The membrane was stained with methylene blue (MB) to visualize the 18S ribosomal RNA (rRNA) as a loading control. The reporter mRNA signals from independent experiments were quantified and normalized to the loading control. Shown is mean ± SD (n = 3).

Source data are available online for this figure.

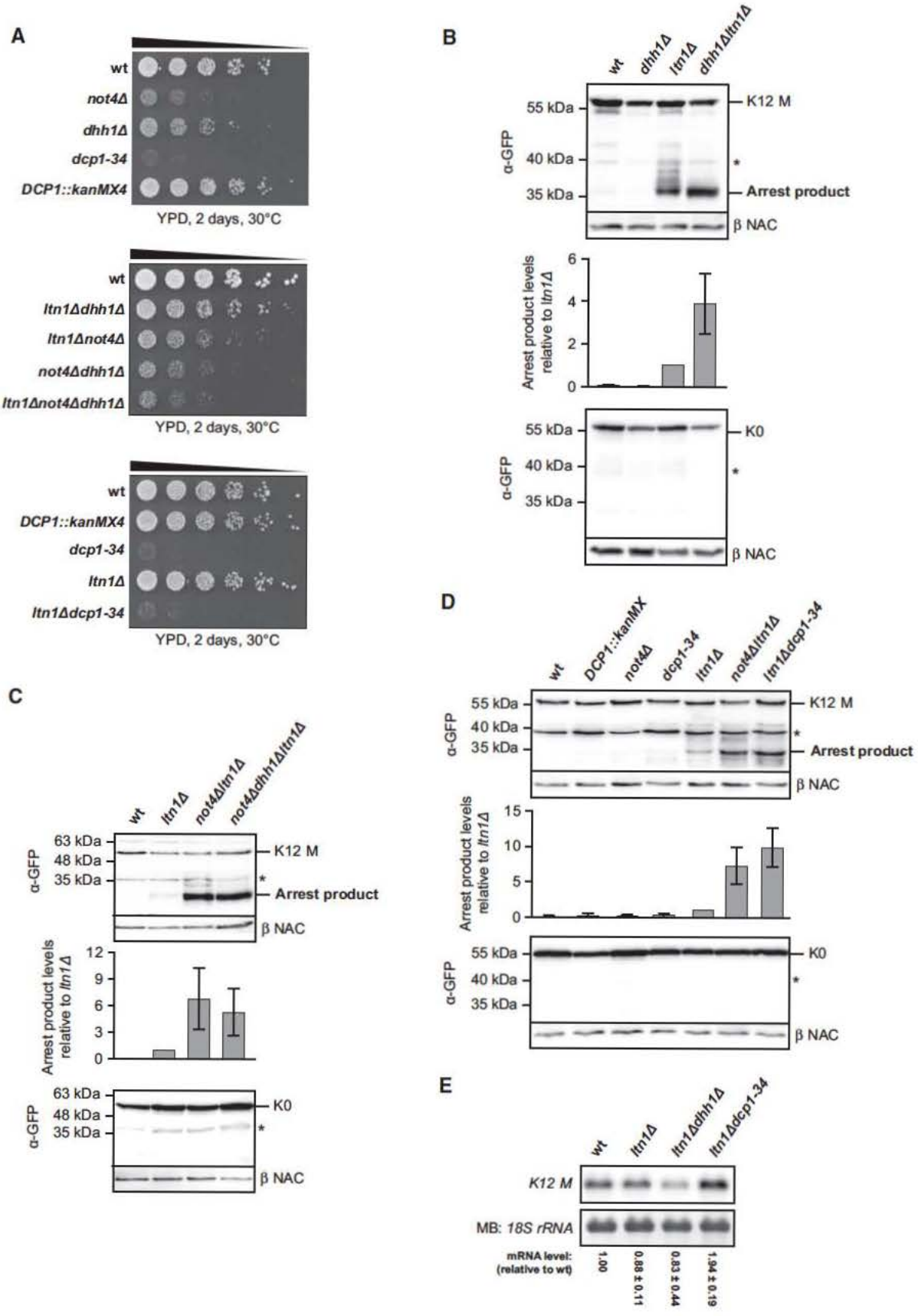
A
wt
not4Δ
dhh1Δ
dcp1-34
DCP1::kanMX4
YPD, 2 days, 30°C
wt
ltn1Δdhh1Δ
ltn1Δnot4Δ
not4Δdhh1Δ
ltn1Δnot4Δdhh1Δ
YPD, 2 days, 30°C
wt
DCP1::kanMX4
dcp1-34
ltn1Δ
ltn1Δdcp1-34
YPD, 2 days, 30°C
B
wt
dhh1Δ
ltn1Δ
dhh1Δltn1Δ
55 kDa
40 kDa
35 kDa
α-GFP
K12 M
*
Arrest product
β NAC
Arrest product levels relative to ltn1Δ
6
4
2
0
55 kDa
40 kDa
35 kDa
α-GFP
K0
*
β NAC
C
wt
ltn1Δ
not4Δltn1Δ
not4Δdhh1Δltn1Δ
63 kDa
48 kDa
35 kDa
α-GFP
K12 M
*
Arrest product
β NAC
Arrest product levels relative to ltn1Δ
12
9
6
3
0
63 kDa
48 kDa
35 kDa
α-GFP
K0
*
β NAC
D
wt
DCP1::kanMX
not4Δ
dcp1-34
ltn1Δ
not4Δltn1Δ
ltn1Δdcp1-34
55 kDa
40 kDa
35 kDa
α-GFP
K12 M
*
Arrest product
β NAC
Arrest product levels relative to ltn1Δ
15
10
5
0
55 kDa
40 kDa
35 kDa
α-GFP
K0
*
β NAC
E
wt
ltn1Δ
ltn1Δdhh1Δ
ltn1Δdcp1-34
K12 M
MB: 18S rRNA
mRNA level: (relative to wt)
1.00
0.88 ± 0.11
0.83 ± 0.44
1.94 ± 0.19

Figure 6.

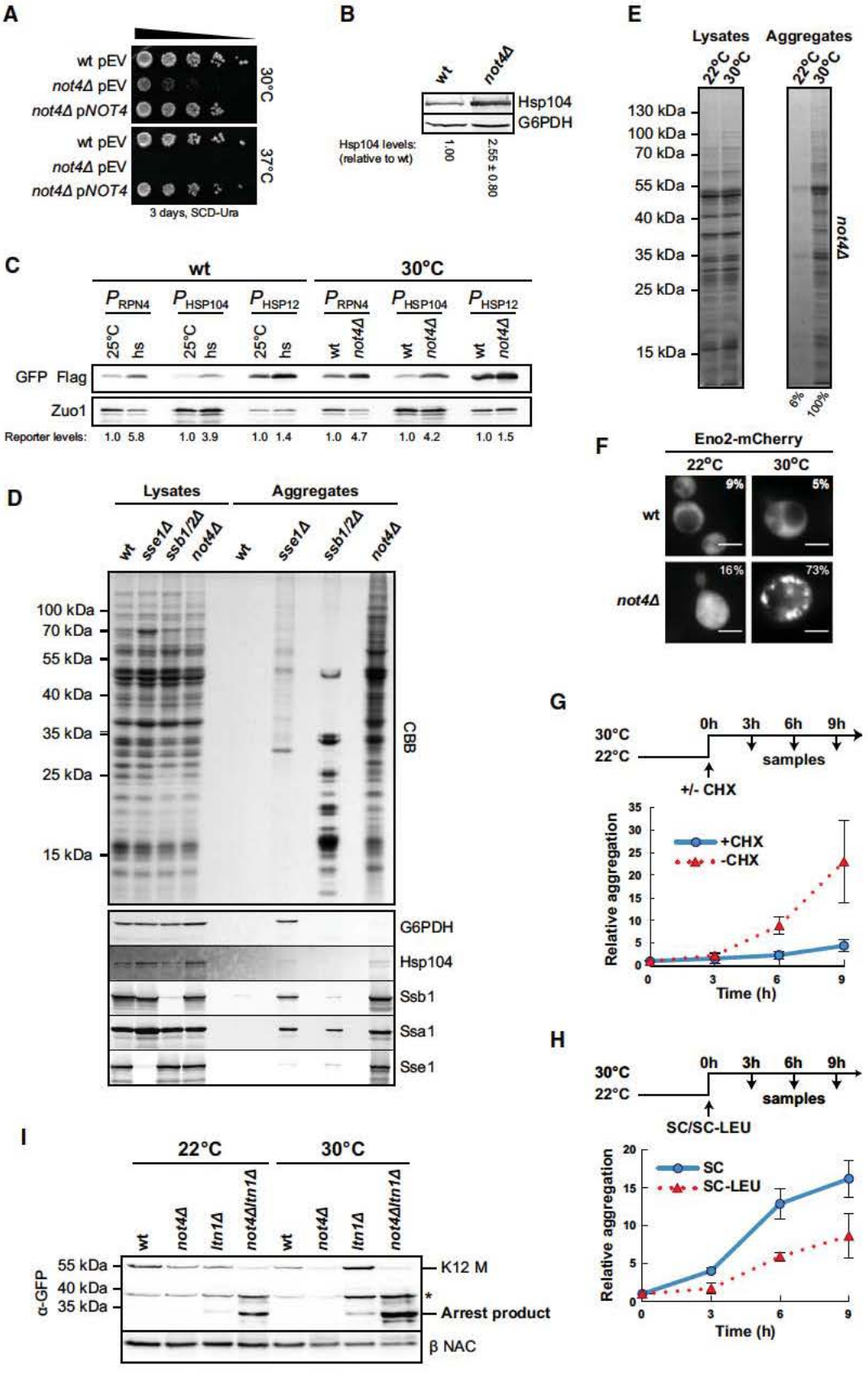
A
wt pEV
not4Δ pEV
not4Δ pNOT4
30°C
wt pEV
not4Δ pEV
not4Δ pNOT4
37°C
3 days, SCD-Ura
B
wt
not4Δ
Hsp104
G6PDH
Hsp104 levels: (relative to wt)
1.00
2.55 ± 0.80
E
Lysates
Aggregates
22°C
30°C
22°C
30°C
130 kDa
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
not4Δ
6%
100%
C
wt
30°C
P RPN4
P HSP104
P HSP12
P RPN4
P HSP104
P HSP12
25°C
hs
25°C
hs
25°C
hs
wt
not4Δ
wt
not4Δ
wt
not4Δ
GFP Flag
Zuo1
Reporter levels:
1.0 5.8
1.0 3.9
1.0 1.4
1.0 4.7
1.0 4.2
1.0 1.5
F
Eno2-mCherry
22°C
30°C
wt
not4Δ
9%
5%
16%
73%
D
Lysates
Aggregates
wt
sse1Δ
ssb1/2Δ
not4Δ
wt
sse1Δ
ssb1/2Δ
not4Δ
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
25 kDa
15 kDa
CBB
G6PDH
Hsp104
Ssb1
Ssa1
Sse1
G
30°C
22°C
0h
3h
6h
9h
samples
+/- CHX
+CHX
-CHX
Relative aggregation
Time (h)
H
30°C
22°C
0h
3h
6h
9h
samples
SC/SC-LEU
SC
SC-LEU
Relative aggregation
Time (h)
I
22°C
30°C
wt
not4Δ
ltn1Δ
not4Δltn1Δ
wt
not4Δ
ltn1Δ
not4Δltn1Δ
α-GFP
55 kDa
40 kDa
35 kDa
K12 M
*
Arrest product
β NAC

Figure 7.

Figure 7. Deletion of *NOT4* affects cellular protein homeostasis.

A Wild type (wt) and *not4Δ* cells with a complementation plasmid (p*NOT4*) or empty vector (pEV) were adjusted to an optical density (OD_{600}) of 0.5, and 5 fold serial dilutions were spotted onto SCD Ura plates. Plates were incubated as indicated.

B Cells were grown at 30°C to the mid log phase, and Hsp104 levels were analysed in normalized lysates by immunoblotting. Glucose 6 phosphate dehydrogenase (G6PDH) was detected as a loading control. Hsp104 signals were quantified and normalized to the loading control. Shown is mean ± SD (n = 4).

C Wt and *not4Δ* cells were transformed with plasmids encoding GFP Flag. Expression was controlled by either one of three different heat shock responsive promoters (*P*) derived from the *HSP104*, *RPN4* and *HSP12* genes (P_{HSP104}, P_{RPN4} and P_{HSP12}). Expression was analysed by Western blotting using Flag specific antibodies. Immunodetection of Zuo1 served as a loading control. The signals were quantified and normalized to the loading control. Left: As a control, wt cells were grown at 25°C to OD_{600} 0.8 and samples were taken before and 40 min after heat shock (hs) at 38°C. Right: Wt and *not4Δ* cells expressing the reporter constructs were grown at 30°C to OD_{600} 0.8 and samples were taken.

D Analysis of protein aggregation in wt and mutant yeast cells. Cells were grown in YPD to OD_{600} 0.8, and protein aggregates were isolated from equal volumes of normalized lysates. The insoluble proteins and samples of the normalized lysates were separated by SDS PAGE and visualized by CBB staining. Bottom: Parallel Western blot analysis of the total and aggregate fractions. G6PDH and the chaperones Hsp104, Ssb1, Ssa1 and Sse1 were detected.

E *not4Δ* cells were grown at 22°C or 30°C to OD_{600} 0.8 and aggregates were analysed as in (D).

F Eno2 Flag mCherry was expressed in wt and *not4Δ* cells at 22°C or 30°C and analysed by fluorescence microscopy. Numbers give the percentage of cells that showed discrete mCherry foci. Scale bars, 5 μm.

G *not4Δ* mutants were grown at 22°C to OD_{600} 0.8 and shifted to 30°C with or without cycloheximide (CHX). Samples were taken at the indicated time intervals and aggregates were isolated for quantification. Mean values and SD bars of three experiments (n = 3) are shown.

H Leucine auxotrophic *not4Δ* mutants were grown at 22°C to OD_{600} 0.8 and shifted to 30°C with or without leucine. Samples were taken and analysed as in (G). Shown is mean ± SD (n = 3).

I Expression of the K12 M reporter at 22°C and 30°C was analysed by Western blotting with antibodies against GFP (α GFP) and β NAC. The asterisk marks a non specific band.

Source data are available online for this figure.

activities and their functional relationship remain elusive. Among those proteins are the two E3 ligases Not4 and Ltn1, which are conserved from yeast to humans and both have proposed functions in the turnover of arrested nascent polypeptides. Whereas Ltn1 is required for efficient cotranslational degradation of arrested nascent chains, we found that Not4 is involved in the negative regulation of translation (Fig 9).

The defects in overall translational repression upon nutrient withdrawal as well as severe translation dependent protein folding stress in the absence of Not4 suggest that Not4 (and proba bly other components of the Ccr4 Not complex) plays a rather general role in translational repression important to maintain protein homeostasis.

Another important observation was that deletion of *NOT4* in combination with *LTN1* in particular increased the expression of translation arrest products. This result is based on the analysis of reporter constructs, which induce strong cotranslational ribosome stalling, for example by consecutive lysine residues. Likewise, ribo somes stall during translation of defective endogenous transcripts before the latter become recognized and eliminated by mRNA surveillance mechanisms (Shoemaker & Green, 2012). Apart from that, ribosome stalling may occur on various non erroneous mRNAs, such as the ones with stable secondary structures, rare codons or regions encoding stretches of positively charged amino acids. Recent ribosome profiling data indicate that translation is rather inhomoge neous (Ingolia *et al*, 2011) and ribosomes stall transiently on many natural transcripts. In any case, ribosome stalling on mRNAs causes jamming of subsequent ribosomes, which leads to the formation of large polysomes. This agrees well with the finding that Not4 and also other subunits of the Ccr4 Not complex were enriched in the late polysomal fractions of sucrose gradients (Fig 1; Supplementary Fig S1) and suggests that they may be specifically recruited to stalled ribosomes to repress further translation of the transcript and to prevent the accumulation of defective proteins (Fig 9).

Not4 is assumed to locate adjacent to the mRNA deadenylases Ccr4 and Caf1 in the Ccr4 Not complex (Bai *et al*, 1999; Basquin *et al*, 2012). Not4 may thus not only repress translation of tran scripts that cause ribosome stalling but also promote their dead enylation and turnover. We indeed observed moderately elevated levels of the ribosome stalling *K12 M* mRNA in cells lacking Not4 (Fig 4A), but our data suggest that differences in mRNA levels have a minor influence on the levels of translation arrest prod ucts (Figs 3 and 4). Moreover, the role of the E3 ligase activity of Not4 in cotranslational protein quality control is still unclear as deletion of *NOT4* enhanced ubiquitination of transiently stalled nascent polypeptides and an E3 ligase deficient mutant fully compensated the loss of Not4 function in translational repression.

To obtain more insights into how Not4 may contribute to translational repression of certain mRNAs, we searched for other proteins that are connected to the Ccr4 Not complex and function in translational repression. The DExD box ATPase and decapping activator Dhh1 meets both criteria. Yeast Dhh1 interacts physically with the Ccr4 Not complex (Hata *et al*, 1998; Coller *et al*, 2001; Maillet & Collart, 2002) and contributes to translational repression (Coller & Parker, 2005). Moreover, an interaction between Dhh1 and polysomes has been described (Drummond *et al*, 2011) and components of the Ccr4 Not complex including Not proteins and Dhh1 localize to cytoplasmic foci called P bodies (Muhlrad & Parker, 2005; Parker & Sheth, 2007), where turnover of transla tionally repressed mRNA takes place. Indeed, we found that also Dhh1 contributes to the repression of K12 protein synthesis (Fig 6B). Since the efficiency of translation initiation depends on intact 5′ mRNA cap structures, it is possible that Not4 exerts its regulatory effect by modulating decapping or cap dependent trans lation initiation through Dhh1 and the decapping enzymes (Fig 9). It is known that the decapping holoenzyme Dcp1 Dcp2 is controlled by decapping activators and some of them recruit Dcp1 Dcp2 to specific transcripts (Li & Kiledjian, 2010). More over, decapping can occur during translation on polysomes (Hu *et al*, 2009, 2010) and thus allows for cotranslational as well as mRNA specific translational repression. In addition, Not2, Not4

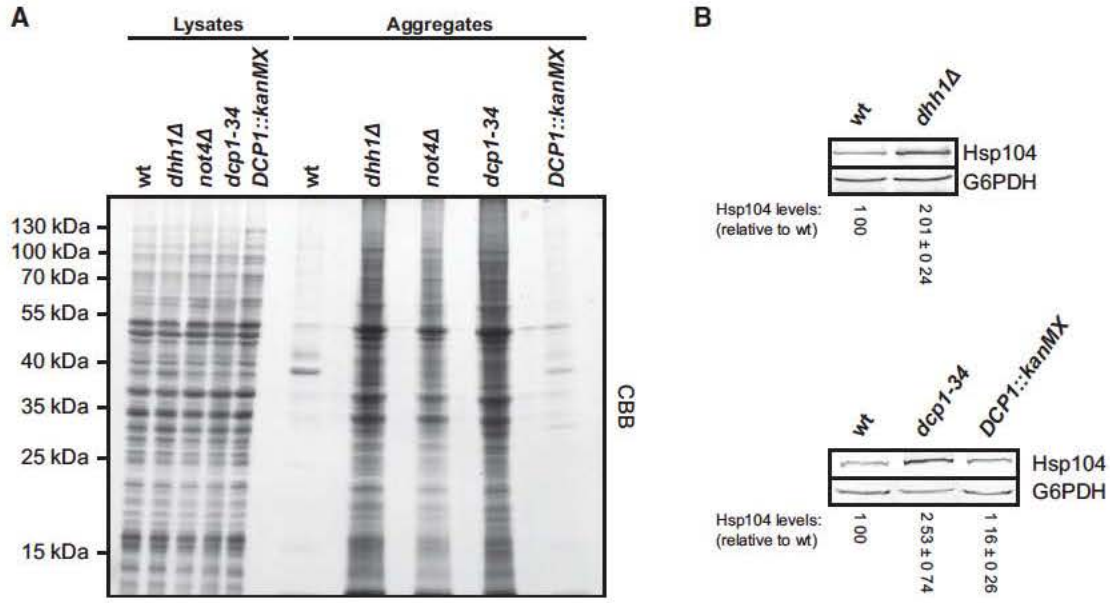

Figure 8. Decapping factors are required for the maintenance of cellular protein homeostasis.

A Protein aggregation was analysed in wild type (wt) and mutant yeast cells. Cells were grown in YPD to an optical density (OD_{600}) of 0.8. Aggregated proteins were isolated from equal volumes of normalized lysates. Insoluble proteins and samples of the normalized lysates were separated by SDS PAGE and visualized by CBB staining.

B Cells were grown to the mid log phase at 30°C, and Hsp104 levels were analysed in normalized lysates by Western blotting. Glucose 6 phosphate dehydrogenase (G6PDH) was detected as a loading control, and Hsp104 signals were quantified and normalized to the loading control. Shown is mean ± SD (n = 4).

Source data are available online for this figure.

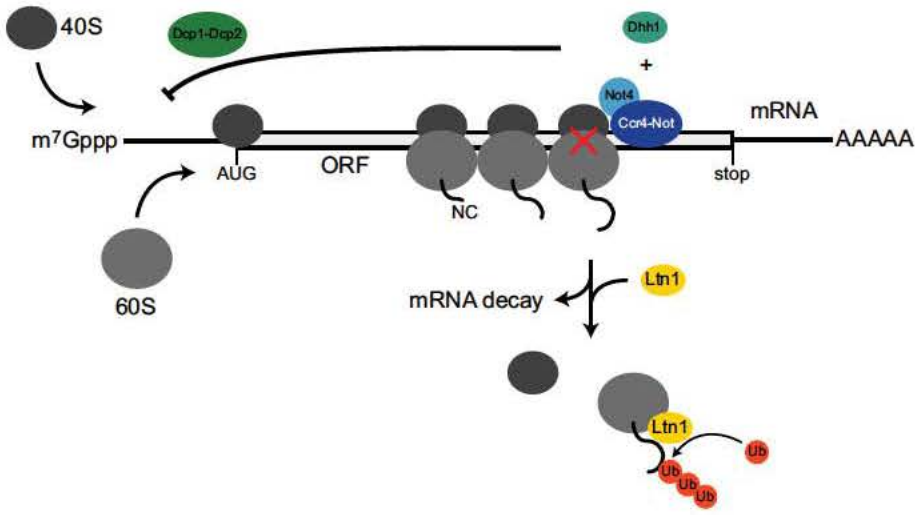

Figure 9. Translational repression of ribosome stalling mRNAs involves Not4, Dhh1 and the decapping factors Dcp1–Dcp2.

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 translational repression. The decapping activator Dhh1 and the decapping proteins Dcp1 Dcp2, which remove the 7 methylguanosine (m^7Gppp) cap structure from the 5′ end of mRNAs, are also required for translational 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.

and Not5 have been implicated in stimulation of deadenylation independent decapping of certain mRNAs (Muhlrad & Parker, 2005), which supports a function of Not4 in translational repression and decapping. This conclusion is further supported by the finding that *dcp1 34* cells show similar defects in translational repression as *not4*Δ and *dhh1*Δ mutants.

Translation initiation and mRNA decapping are competing processes. Accordingly, decapping can be directly inhibited by cap binding translation initiation factors which dissociate from transcripts before decapping occurs (Schwartz & Parker, 1999, 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 translational repression may occur more directly on the level of translation initiation, for example 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 translation elongation (Sweet *et al*, 2012).

Not4 and the Ccr4 Not complex repress translation of specific mRNAs in the germline of fruit flies (Kadyrova *et al*, 2007). In this and other cases, the Ccr4 Not complex is recruited to the 3′ UTRs of target mRNAs via pumilio family proteins (Goldstrohm *et al*, 2006; Van Etten *et al*, 2012). In animal cells, the Ccr4 Not complex acts in microRNA mediated translational repression and deadenylation of specific transcripts (Cooke *et al*, 2010; Braun *et al*, 2011; Chekulaeva *et al*, 2011; Fabian *et al*, 2011) involving also Dhh1 orthologs (Chen *et al*, 2014b; Mathys *et al*, 2014; Rouya *et al*, 2014). The principal function of Ccr4 Not components in translational repression seems thus to be conserved. However, ribosome stalling dependent translational repression in yeast cannot be explained by targeting of Not4 to specific sequences in the 3′ UTR and thus requires other recruitment signals. For example in metazoans, the translational repressor protein FMRP, which shows a similar distribution in polysome profiles as Not4 (Darnell *et al*, 2011), first binds to its target mRNAs and later during translation directly to ribosomes, where it likely inhibits elongation through steric effects (Chen *et al*, 2014a). The FMRP ribosome interaction involves RNA binding motifs. Not4 also contains 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 (Fig 1C), indicating different interaction characteristics. In addition, Not4 recruitment could be mediated by another component of the Ccr4 Not complex. 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.

Deletion of either the *NOT4* or *DHH1* gene as well as a *DCP1* mutation caused translational misregulation, constitutive folding stress and strong protein aggregation. These very similar defects in the different mutants suggest that: (i) Not4, Dcp1 and Dhh1 act in the same pathway, and (ii) that the loss of negative control during protein synthesis is causative for the proteostatic imbalance in these cells. The observation that aggregation in *not4*Δ cells can be ameliorated by inhibition of protein synthesis supports this assumption. Interestingly, we did not observe severe aggregation in cells lacking Ltn1 or in mutants with defective mRNA decay, suggesting that efficient backup systems exist that can substitute for these activities. In contrast, cells lacking Not4 cannot suppress protein aggregation although the heat shock response is induced, emphasizing the unique function of Not4 in translational quality control and its importance for cellular protein homeostasis.

Materials and Methods

Yeast strains and growth conditions

All yeast strains used in this study were isogenic derivatives of BY4741 and BY4743 (Brachmann *et al*, 1998). Unless described otherwise, cells were grown under standard conditions at 30°C in YPD [1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, 2% (w/v) dextrose] or defined synthetic complete (SC) media (6.7 g/l Bacto yeast nitrogen base without amino acids, 2 g/l SC amino acid mix) containing 2% (w/v) glucose (SCD) (Guthrie & Fink, 2002; Amberg *et al*, 2005). Spot assays were performed by adjusting yeast cultures to the same optical density (OD_{600}), and fivefold serial dilutions were spotted onto agar plates. Plates were incubated as indicated.

Polysome profiling

Yeast cells were grown at 30°C in YPD medium to OD_{600} 1.0. The culture was poured on crushed ice and harvested by centrifugation at 4°C in the presence of 100 μg/ml cycloheximide to stabilize translating ribosomes. Lysates were prepared by glass bead disruption (FastPrep 24, MP) of the yeast cells in lysis buffer [20 mM HEPES KOH pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), 100 μg/ml cycloheximide, 1× Complete protease inhibitor cocktail (Roche)]. Afterwards, the lysates were cleared twice by centrifugation at 16,000 *g* at 4°C for 10 min and absorbance values at 260 nm (A_{260}) were normalized with lysis buffer. Volumes of each lysate equivalent to eight A_{260} absorbance units were loaded onto an 11 ml 15 45% (w/v) sucrose gradient prepared with a gradient forming instrument (Gradient Master, Biocomp Instruments) in lysis buffer without PMSF and centrifuged in a TH 641 rotor (Sorvall) at 39,000 rpm for 2 h at 4°C. Upon centrifugation, the gradients were fractionated from the top with a gradient fractionator (Teledyne Isco, Inc.) and the A_{254} signals were recorded to detect the fractions containing soluble proteins, ribosomal subunits, 80S monosomes as well as polysomes. 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. The collected fractions were precipitated with trichloroacetic acid (TCA), and the proteins were separated by SDS PAGE followed by Western blotting. Larger gradients were prepared where indicated. For that, volumes equivalent to 180 200 A_{260} units were loaded onto a 38 ml 10 40% (w/v) sucrose gradient and centrifuged in a SW28 rotor (Beckman) at 25,000 rpm for 7 h at 4°C. The readout was performed as described above. To disrupt polysomes, the lysates were treated with RNase A (Fermentas) at a final concentration of 300 μg/ml and incubated for 15 min on ice prior to density gradient centrifugation.

Analysis of translation arrest *in vivo*

Yeast cells were transformed with plasmids encoding ribosome stalling reporter proteins and grown to the exponential phase in SCD His medium. Cells were lysed by glass bead disruption in lysis buffer (50 mM HEPES KOH pH 7.4, 100 mM potassium acetate, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2× Complete protease inhibitor mix) and analysed by SDS PAGE and Western blotting.

Nutrient withdrawal experiments

Nutrient withdrawal experiments were performed as described in Ashe *et al* (2000) and Holmes *et al* (2004) with minor modifications. To analyse translational repression upon glucose depletion, yeast cells were grown in 1 l YPD to OD_{600} 0.5. The culture was split and cells were sedimented at 1,000 *g* for 3 min at 30°C. The cells were then resuspended in 500 ml pre warmed YP with or without 2% (w/v) glucose and incubated at 30°C. After 10 min translation was stopped with 100 μg/ml cycloheximide and cells were rapidly chilled by pouring the culture on crushed ice. The cells were harvested for polysome profiling as described above. Amino acid depletion was performed likewise except that cells were grown in SCD and resuspended in pre warmed starvation medium [6.7 g/l Bacto yeast nitrogen base without amino acids, 2% (w/v) glucose] with or without 2 g/l SC amino acids.

Measurement of translation activity upon glucose withdrawal

Measurement of translation activity upon glucose depletion was performed as previously described (Ashe *et al*, 2000). Cells were grown in 20 ml SCD medium to OD_{600} 0.5 at 30°C. A total of 2 OD_{600} units of cells were sedimented for 2 min at 1,000 *g* and resuspended in 10 ml pre warmed labelling medium [SC Met, 59.5 ng/ml methionine, 0.5 ng/ml ^{35}S methionine (1,000 Ci/mmol, Hartmann Analytic)] with or without 2% (w/v) glucose.

Isolation of protein aggregates

Aggregates were prepared as described previously (Koplin *et al*, 2010). Liquid yeast cultures were inoculated with stationary cells to OD_{600} 0.1. Cells were grown in YPD at 30°C to OD_{600} 0.8 1.0, harvested in 50 ml aliquots in the presence of 15 mM sodium azide and flash frozen in liquid nitrogen. For cell lysis, the frozen pellets were resuspended in 1 ml buffer I [20 mM potassium phosphate pH 6.8, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% (v/v) Tween 20, protease inhibitors, 1 mM PMSF, 1.25 U/ml DNase (Sigma)] containing 3 mg/ml Zymolyase T20 (MP Biomedicals), incubated for 20 min at room temperature and chilled on ice. Upon sonication (Branson tip sonifier; eight times at level 4 and 50% duty cycle), the samples were centrifuged for 20 min at 200 *g* and the protein concentrations of the supernatants were normalized in a final volume of 800 μl. A sample of each normalized lysate was taken as an input control. The aggregated proteins were sedimented at 16,000 *g* for 20 min. The pellets were washed twice with buffer II [20 mM potassium phosphate (pH 6.8), protease inhibitors] containing 2% (v/v) Nonidet P 40, sonicated (six times at level 4 and 50% duty cycle) and centrifuged at 16,000 *g* for 20 min at 4°C. Finally, the aggregated proteins were washed in buffer II, suspended in sample buffer and separated by SDS PAGE. The proteins were visualized by Coomassie staining and quantified using ImageJ64 (NIH).

Protein synthesis-dependent aggregation

To study protein synthesis dependent aggregation, *not4*Δ cells were grown at 22°C to OD_{600} 0.8 in YPD medium. A total of 300 μg/ml cycloheximide was added to half of the cells to stop protein synthesis, while the other half remained untreated. Both cultures were shifted to 30°C to induce protein aggregation. Samples were taken at different time points and aggregated proteins were extracted as described above. The aggregated proteins were separated by SDS PAGE and quantified. Alternatively, *not4*Δ cells were grown at 22°C to OD_{600} 0.8 in YPD medium and washed twice with sterile water. The cells were then resuspended in SCD medium with or without leucine (SCD Leu) and shifted to 30°C (note that the yeast cells were leucine auxotrophic). Samples were taken at different time intervals and protein aggregates were isolated.

Ribosome cosedimentation assay

For the release of nascent peptides from ribosomes, yeast cells were grown in SCD medium without uracil (SCD Ura) to OD_{600} 1 and 40 OD_{600} units were collected by centrifugation. The cells were washed in SCD medium lacking uracil and methionine (SCD Ura Met), resuspended in SCD Ura Met and starved for 45 min at 30°C. To label nascent polypeptide chains and newly synthesized proteins, 20 μCi/ml of ^{35}S methionine (Hartmann Analytik) was added to the cells for 50 s. Afterwards, the cells were chilled on ice. Half of the cells were treated with 300 μg/ml cycloheximide to stabilize ribosome nascent chain complexes, whereas the other half was incubated with 0.1 mM puromycin for 5 min on ice to release nascent polypeptides from ribosomes. The cells were then pelleted and lysed by glass bead disruption in buffer III (50 mM HEPES KOH pH 7.4, 100 mM potassium acetate, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2× Complete protease inhibitor cocktail) with or without 300 μg/ml cycloheximide. The lysates were cleared by centrifugation at 16,000 *g* for 10 min at 4°C, protein concentrations were normalized, and a sample was taken. The lysate from the puromycin treated cells was again incubated for 1 h with 0.6 mM puromycin on ice to increase the efficiency of nascent chain release. Next, equal volumes of the lysates were loaded onto a 20% (w/v) sucrose cushion prepared in buffer III with or without 300 μg/ml cycloheximide, respectively, and centrifuged for 90 min at 200,000 *g* at 4°C to sediment the ribosomes. Upon centrifugation, the ribosomes were resuspended in lysis buffer. The A_{260} values of the ribosome solutions were normalized and equal volumes were supplemented with SDS sample buffer. Volumes equivalent to 1.1 A_{260} units of ribosomes as well as 20 μg of the total samples were loaded per lane onto a gel and analysed by SDS PAGE and Western blotting. To confirm the release of nascent chains by puromycin treatment, radioactive signals in the ribosomal pellet fractions were detected by autoradiography with the FLA 9000 system (Fujifilm). To analyse the ribosome association of translation arrest products, yeast cells were transformed with plasmids encoding the K12 M ribosome stalling construct and the cells were cultured to the exponential phase in SCD His medium. Cell lysates were prepared by glass bead disruption in lysis buffer (20 mM HEPES KOH pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM PMSF, 2× protease inhibitor mix). Volumes of lysates equivalent to 1.5 A_{260} units were diluted to 200 μl and loaded onto a 600 μl sucrose cushion [25% (w/v) sucrose in lysis buffer]. Ribosomes were pelleted by ultracentrifugation at 200,000 *g* for 90 min (rotor S140 AT; Sorvall) at 4°C and resuspended in SDS sample buffer. Samples of the normalized lysates and supernatants were precipitated with TCA and resuspended in alkaline SDS sample buffer. Aliquots of each fraction were analysed by SDS PAGE and Western blotting.

Immunoprecipitation (IP)

Denaturing IP of K12 proteins was performed as described in Bengtson and Joazeiro (2010). Yeast cells were transformed with the plasmid p413GPD GFP 2A FLAG HIS3 K12 and grown overnight in SCD His medium containing 0.17% (w/v) yeast nitrogen base without ammonium sulphate and 0.1% (w/v) proline. The cells were used to inoculate fresh medium containing 0.003% (w/v) SDS to OD_{600} 0.5 and grown for 3 h at 30°C. Afterwards, 75 μM MG132 was added for 30 min (Liu *et al*, 2007) and cells were collected. The cells were then incubated 5 min in 0.1% (w/v) NaOH at room temperature, pelleted, resuspended in lysis buffer [1% (w/v) SDS, 50 mM Tris HCl pH 7.4, 5 mM EDTA, 5 mM *N* ethylmaleimide (NEM), 10 μg/ml aprotinin, 5 μg/ml leupeptin, 8 μg/ml pepstatin A] and boiled. The cleared lysates were adjusted to a protein concentration of 4 μg/μl in 100 μl lysis buffer, diluted with 900 μl IP buffer A [50 mM Tris HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.5% (v/v) NP40] and incubated with 25 μl anti FLAG M2 magnetic beads (Sigma) for 2 h at 4°C. The beads were washed three times with IP buffer A, and bound proteins were eluted by boiling in 2× SDS sample buffer. Samples of the adjusted lysates and the IP eluates were analysed by Western blotting. Ubiquitinated proteins were detected with polyclonal anti ubiquitin antibodies (Dako).

Northern blotting

Yeast cells were transformed with reporter plasmids and grown in SCD His medium to OD_{600} 0.8. Total RNA was extracted with the hot phenol method (Schmitt *et al*, 1990) followed by ethanol precipitation. Equal amounts of RNA were separated by agarose gel electrophoresis and transferred to a Biodyne A membrane (Pall Life Sciences). The membrane was stained with methylene blue to confirm equal loading. Digoxigenin (DIG) labelled RNA probes were synthesized with the DIG RNA Labeling Kit (Roche), and mRNA was detected with reagents provided by the DIG Northern Starter Kit (Roche). The signals on the blots were detected with the LAS 3000 system (Fujifilm). The oligonucleotides 5′ GAACTCTTCACTGGAG TTGTCC 3′ and 5′ gatcTAATACGACTCACTATAGGGgtttgtctgccatga tgtatac 3′ were used to amplify template DNA for the T7 based *in vitro* synthesis of DIG labelled *GFP* RNA probe. To determine mRNA half lives, the *K0* and *K12 M* reporter mRNAs were expressed under control of a galactose inducible promoter (*GAL1*) on a centromeric plasmid [pRS316(GAL1) GFP Flag HIS3 and pRS316(GAL1) GFP K12 Flag HIS3], respectively. The cells were grown in SC Ura medium containing 2% (w/v) galactose for steady state expression of the reporter mRNAs to OD_{600} 0.8 and transferred to SCD Ura medium [supplemented with 2% (w/v) glucose] for transcriptional shut off. Samples were taken at different time intervals and RNA was extracted for Northern blotting. The Northern blot signals were quantified and normalized to the 18S ribosomal RNA. Half lives were determined by non linear regression analysis using Prism (GraphPad Software, Inc.) and calculated as described in Coller (2008) with the equation $t_{1/2} = \ln(2)/k$, where k = rate constant for mRNA decay.

Stress reporter assay

Yeast cells were transformed with the plasmids p413P_{RPN4} GFP Flag, p413P_{HSP12} GFP Flag and p413P_{HSP104} GFP Flag, respectively. Single clones were isolated and grown in SCD His medium to OD_{600} 0.8 at 25°C. To test the responsiveness of the reporter constructs to heat shock, a sample was taken from wild type cells carrying the different plasmids before and after a temperature shift to 38°C for 40 min. To test for constitutive expression of the stress reporters in the wild type and *not4*Δ strain, stationary overnight cultures were used to inoculate SCD His medium to OD_{600} 0.15 and the cultures were grown at 22°C or 30°C until OD_{600} 0.8 was reached. Afterwards, the cells were pelleted, resuspended in FPB [50 mM HEPES KOH pH 7.4, 100 mM potassium acetate, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 2× Complete protease inhibitor cocktail (Roche)] on ice and lysed by glass bead disruption. The lysates were cleared by centrifugation at 16,000 *g* for 2 min, and the protein concentrations were determined (Protein Assay, Bio Rad). Fifteen micrograms of each lysate were loaded on a SDS gel. Expression of the GFP Flag reporter constructs was detected by Western blotting.

Microscopy

For standard and fluorescence microscopy, yeast cells were grown in YPD to OD_{600} 1 at 22°C or 30°C. Then, the cells were fixed by the addition of 4% (v/v) formaldehyde for 10 min and one OD_{600} unit of cells was pelleted. Microscopy was performed with a Visitron microscope (37081 Visitron Systems, Axio, Carl Zeiss Inc.) equipped with a 100× magnification Plan Apochromat oil objective. All images were recorded at room temperature and in PBS (pH 7.4) as imaging medium. Fluorescence was observed using a FITC filter. Pictures were recorded with the Spot Pursuit camera (model 23.0) and 1.4 MP monochrome without irradiation. VisiView (Visitron Systems) was used as an acquisition software, and the images were processed with Photoshop CS3 (Adobe) and ImageJ64 (NIH).

Protein turnover experiments

The protocol for the measurement of protein turnover was adapted from Medicherla and Goldberg (2008) and Seufert and Jentsch (1990). Cells were grown in YPD to the exponential phase (OD_{600} 0.5 0.8) at 30°C, washed and starved for 60 min in SCD Met medium. 20 μCi/ml ^{35}S methionine was added for 5 min to label newly synthesized proteins. Protein synthesis was stopped with 0.5 mg/ml cycloheximide, cells were washed twice in ice cold chase medium (SCD containing 0.5 mg/ml cycloheximide and 1 mg/ml methionine) and incubated in pre warmed chase medium at 30°C. Samples were taken at different time intervals, and protein degradation was stopped by mixing with TCA at a final concentration of 10% (w/v) on ice. Radioactivity in the total sample and sample supernatant was determined by liquid scintillation counting. Protein degradation is given as the TCA soluble fraction of total incorporated radioactivity released from cells during the chase period relative to $t = 0$.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

SP conceived the study, designed the experiments, performed the research, analysed the data and prepared the manuscript. JR performed the experi ments, contributed to experimental design and analysed the data. MK performed the experiments, contributed to experimental design and analysed the data. AS performed the experiments and analysed the data. MB performed the experiments. TF analysed mass spectrometry data. ED directed the study, contributed to experimental design, data analysis and manuscript preparation and submitted the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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