Reviewed Preprint
v1 • June 23, 2026
Not revised

For correspondence:
ulrike.kutay@bc.biol.ethz.ch

Competing interests: No competing interests declared

Funding: See page 16

Reviewing editor: Ivan Topisirovic, McGill University, Canada

RNF25 is activated as a response to amino acid starvation-induced ribosome collisions in competition with GCN2

Ivan Kisly, Ivo Zemp, Ulrike Kutay
Institute of Biochemistry, Department of Biology, ETH Zurich, Zurich, Switzerland

eLife Assessment

This study provides conditionally **useful** evidence that amino acid starvation and other stresses induce RNF25-dependent ubiquitination of RPS27A/eS31, extending this pathway beyond A-site-trapping conditions and implicating GCN1. However, **incomplete** and largely indirect evidence was provided to support key mechanistic claims-notably competition between RNF25 and GCN2 for GCN1 and a role in resolving ribosome collisions. Additional direct and orthogonal evidence is required to substantiate these conclusions.

https://doi.org/10.7554/eLife.112045.1.sa4

Abstract

Surveillance of mRNA translation relies on a suite of ribosome-associated quality control pathways. Recently, a novel pathway induced by trapping of translation factors in the ribosomal A-site has been described, involving ubiquitination of RPS27A/eS31 by the human E3 ubiquitin ligase RNF25. Here, we show that not only ribosome-stalling by low doses of translation inhibitors, but also amino acid starvation induces RPS27A/eS31 ubiquitination, identifying a natural trigger of RNF25 activation. Even under optimal growth conditions, RNF25 senses and resolves transient ribosome stalls. RPS27A/eS31 ubiquitination specifically depends on the ribosome collision sensor GCN1, a known cofactor of GCN2 involved in the integrated stress response. RNF25 and GCN2 both possess a GCN1-binding RWD domain, indicating a competitive relationship, with GCN2 acting as a negative regulator of RNF25 activation. Although both RNF25 and GCN2 respond to amino acid starvation, RPS27A/eS31 ubiquitination by RNF25 is not required for GCN2 activation, showing that both act in independent pathways. We propose that the RNF25 pathway acts as a first line of defence to resolve ribosome collisions, outcompeted by GCN2 binding to GCN1 under acute stress.

Introduction

Protein synthesis is a fundamental biological process in which ribosomes translate genetic information encoded in mRNA into proteins. Since proteins participate in every aspect of cell life, alterations in their synthesis can disturb cellular homeostasis. For example, defects in the resolution of stalled or collided ribosomes have been associated with a wide range of pathologies, such as neurodegeneration and mitochondrial dysfunction (Choe *et al*, 2016; Chu *et al*, 2009; Defenouillere *et al*, 2016; Geng *et al*, 2024; Izawa *et al*, 2017; Martin *et al*, 2020; Udagawa *et al*, 2021; Wu *et al*, 2019b). To avoid such negative scenarios, cells have evolved a vast number of surveillance pathways that control protein synthesis (Chang & Choe, 2026; Ford *et al*, 2024; Joazeiro, 2019; Park *et al*, 2021; Yip & Shao, 2021).

Ribosome stalling can be caused by a variety of conditions, such as translation inhibitors, mRNA damaging reagents or UV radiation, the production of aberrant mRNAs, the presence of specific mRNA stalling motifs, or amino acid starvation (Chang & Choe, 2026; Park *et al*., 2021). Three major pathways recognize and handle ribosome collisions: the ribosome-associated quality control (RQC), the integrated stress response (ISR) and the ribotoxic stress response (RSR) (Chang & Choe, 2026; Park *et al*., 2021). The RQC pathway serves as a first line of defence dealing with a basal level of collisions that may occur even under optimal growth conditions. Here, collided ribosomes are recognized by the core RQC factor ZNF598 (or the yeast homolog Hel2) (Juszkiewicz *et al*, 2018; Juszkiewicz & Hegde, 2017; Sundaramoorthy *et al*, 2017). Independently of that, the collision sensor EDF1 recruits GIGYF2-4EHP to the stalled ribosomes, which blocks translation initiation to reduce usage of the affected mRNAs (Juszkiewicz *et al*, 2020a; Sinha *et al*, 2020). These events promote the engagement of factors responsible for the splitting of ribosomes into 40S and 60S subunits as well as degradation of aberrant mRNAs and nascent polypeptides. Upon more global translational stress causing more persistent collisions, the ISR pathway is additionally activated through the recognition of collided ribosomes by GCN1 and subsequent recruitment of the kinase GCN2 to the stalling ribosome. Active GCN2 phosphorylates eIF2α, causing both the inhibition of translation and transcriptional activation of stress response genes (Castilho *et al*, 2014; Darnell *et al*, 2018; Harding *et al*, 2019; Hinnebusch, 2005; Ishimura *et al*, 2016; Marton *et al*, 1993; Pochopien *et al*, 2021; Wu *et al*, 2020; Zhou *et al*, 2025). Finally, upon severe and persistent translational stress, the RSR pathway is induced by the MAP3K ZAKα, which becomes activated on collided ribosomes and initiates the activation of pro-inflammatory and pro-apoptotic pathways (Huso *et al*, 2026; Sinha *et al*, 2024; Stoneley *et al*, 2022; Vind *et al*, 2020). It is important to note, that although these three pathways may be considered independent processes, they have complex antagonistic as well as cooperative relations with each other (Chang & Choe, 2026; Kim *et al*, 2024; Park *et al*., 2021; Pochopien *et al*., 2021).

An important role in surveillance pathways is assigned to protein ubiquitination, which facilitates protein degradation but also has many regulatory functions (Dougherty *et al*, 2020; Ford *et al*., 2024). For example, recent studies showed that regulatory ribosomal ubiquitination is a crucial step in the activation of the RQC pathway (Juszkiewicz & Hegde, 2017; Sundaramoorthy *et al*., 2017). Collided ribosomes induce ubiquitination of the ribosomal proteins RPS10/eS10 and RPS20/uS10 by the E3 ubiquitin ligase ZNF598, which is antagonized by deubiquitinases (DUBs) USP21 and OTUD3 (Garshott *et al*, 2020; Ikeuchi *et al*, 2019; Juszkiewicz *et al*., 2018; Juszkiewicz & Hegde, 2017; Sundaramoorthy *et al*., 2017). Ubiquitination of ribosomal proteins in RQC is needed to recruit the ASCC complex, which facilitates splitting of stalled ribosomes (Best *et al*, 2023; Hashimoto *et al*, 2020; Juszkiewicz *et al*, 2020b; Matsuo *et al*, 2017; Matsuo *et al*, 2023; Narita *et al*, 2022). However, despite advances in understanding how ubiquitination acts in translational stress, many aspects and targets of such modifications remain uncharacterized, and regulatory ribosomal ubiquitination remains an important topic for both basic science and medical research.

Recently, we discovered a novel translation-dependent mono-ubiquitination event targeting RPS27A/eS31 in human cells (Fig 1A) (Montellese *et al*, 2020). We demonstrated that RPS27A/eS31 is modified on lysine K113, which is counteracted by the DUB USP16 (Montellese *et al*., 2020). Further work in the field then revealed that RPS27A/eS31 ubiquitination is mediated by the E3 ligase RNF25 as a part of a novel RQC pathway, which is induced in response to treatment of cells with the translation inhibitors ternatin-4, SRI-41315 or NVS1.1 (Gurzeler *et al*, 2023; Oltion *et al*, 2023). These drugs bind the translation factors eEF1A (in case of ternatin-4) and eRF1 (in case of SRI-41315 and NVS1.1), respectively, and trap them in the A-site of the ribosome (Gurzeler *et al*., 2023; Oltion *et al*., 2023). Affected ribosomes stall on mRNA, which causes ribosome collisions that are sensed by GCN1. This leads to RPS27A/eS31 ubiquitination and subsequent recruitment of the E3 ubiquitin ligase RNF14 for ubiquitination and clearance of the trapped translation factors from the ribosome (Gurzeler *et al*., 2023; Oltion *et al*., 2023).

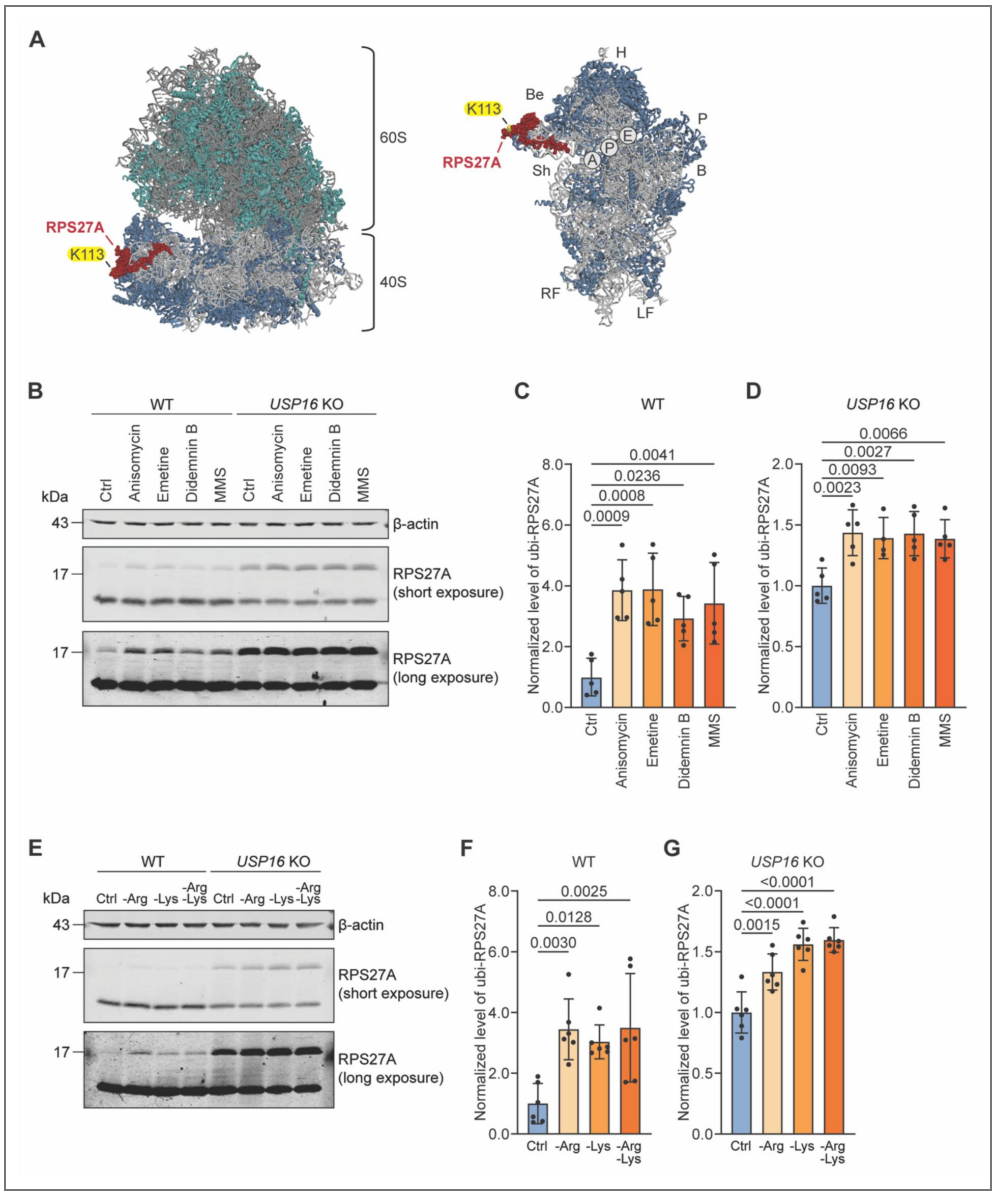

Figure 1. Ubiquitination of RPS27A/eS31 is induced by conditions that cause ribosome stalling including amino acid starvation.

(A) Structural models of the human 80S ribosome (left) and of the respective 40S subunit (right) (PDB ID: 6QZP, (Natchiar *et al*, 2017)). Ribosomal protein RPS27A/eS31 (red) as well as its K113 (yellow) are indicated. 80S is shown from the P-stalk side, 60S subunit (rRNA in dark grey and proteins in teal) and 40S subunit (rRNA in light grey and proteins in blue) are indicated. On the right, 40S is shown from the subunit interface side. Positions of A-, P-, and E-sites as well as major structural elements of the 40S are indicated (B, body; Be, beak; H, head; LF, left foot; RF, right foot; Sh, shoulder). **(B)** Induction of RPS27A/eS31 ubiquitination by diverse ribosome-stalling compounds. HeLa WT and *USP16* KO cells were cultivated in the absence or presence of the following drugs: 0.19 µM anisomycin (30 min), 1.8 µM emetine (30 min), 0.5 µM didemnin B (30 min, 0.25 µg/ml MMS (60 min). Cell extracts were analysed by immunoblotting with the indicated antibodies. **(C, D)** Quantification of levels of ubiquitinated RPS27A/eS31 in HeLa WT C) and *USP16* KO (D) lysates from (B), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to samples from untreated cells (N ≥ 4, mean ± SD, one-way ANOVA and post hoc Dunnett's test; p-values are indicated). **(E)** Amino acid starvation enhances RPS27A/eS31 ubiquitination. HeLa WT and *USP16* KO cells were grown for 6 h in control conditions (Ctrl, no starvation), or in the absence of arginine (-Arg), lysine (-Lys) or both (-Arg-Lys). Cell extracts were analysed by immunoblotting with the indicated antibodies. **(F, G)** Quantification of levels of ubiquitinated RPS27A/eS31 in HeLa WT (F) and *USP16* KO (G) lysates from (E), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to samples from non-starved cells (N = 6, mean ± SD, one-way ANOVA and post hoc Dunnett's test, p-values are indicated.

Here, we have investigated the triggers and the underlying mechanism of the RNF25 pathway in more detail. Our analysis revealed that sub-inhibitory concentrations of several translation inhibitors induce activation of RNF25. We identify amino acid starvation as a physiological stalling condition that induces ubiquitination of RPS27A/eS31. Both compound- and starvation-induced RPS27A/eS31 ubiquitination is specifically dependent on the collision sensor GCN1, but not on EDF1 or ZAKα, establishing RPS27A/eS31 ubiquitination as part of a distinct pathway. Since GCN1 is known to be involved in the ISR, we evaluated the relation of RPS27A/eS31 ubiquitination in relation to stress signalling by GCN2. Exploiting cells harbouring a ubiquitination-deficient K113R mutant, we show that RNF25 is not required for GCN2 activation, establishing them as factors acting independently downstream of GCN1. In support of this model, RNF25 gets hyperactivated in the absence of GCN2, suggesting that both pathways co-exist as two parallel pathways dependent on GCN1, guarding cells from ribosome stalling.

Results

Ubiquitination of RPS27A/eS31 is induced by conditions that cause ribosome stalling

Several recent studies have described ubiquitination of RPS27A/eS31 as a part of a protein synthesis quality control pathway activated in the presence of drugs that trap eEF1A or eRF1 in the A-site of the ribosome, causing stalling and subsequent collision of ribosomes (Gurzeler *et al*., 2023; Oltion *et al*., 2023). Here, we aimed at better understanding the mechanism of RPS27A/eS31 ubiquitination. First, we tested whether we could detect ubiquitination of RPS27A/eS31 upon treatment of HeLa WT cells with ternatin, a commercially available but slightly less potent analogue of ternatin-4 (Carelli *et al*, 2015). Quantitative immunoblotting indeed revealed increased levels of ubiquitinated RPS27A/eS31 after ternatin treatment (Fig S1A, S1B), consistent with previous work (Oltion *et al*., 2023). However, the levels of ubiquitinated RPS27A/eS31 in WT cells are relatively low, hampering a robust quantitative assessment of this modification due to a low signal-to-noise ratio (Fig S1A, S1B). To confirm our results and to circumvent this issue, we therefore analysed RPS27A/eS31 ubiquitination in HeLa *USP16* KO cells. Due to the lack of USP16, the DUB for RPS27A/eS31 (Montellese *et al*., 2020), these cells naturally contain higher levels of ubiquitinated RPS27A/eS31, which are further increased upon induction of stalling, facilitating quantitative analyses (Fig S1A, S1C). Indeed, ternatin increased the levels of ubiquitinated RPS27A/eS31 in these cells about 1.5-fold.

To understand whether other ribosome stalling conditions can also induce ubiquitination of the RPS27A/eS31, HeLa WT and *USP16* KO cells were treated with drugs known to stall ribosomes through various mechanisms, i.e. anisomycin, emetine, didemnin B and methyl methanesulfonate (MMS) (Fig 1B-D). Anisomycin binds to the 60S ribosomal subunit and prevents peptide bond formation (Garreau de Loubresse *et al*, 2014). Emetine binds to the 40S ribosomal subunit and blocks tRNA translocation (Wong *et al*, 2014). Didemnin B acts similarly to ternatin, trapping the translation elongation factor eEF1A on the ribosome (Juette *et al*, 2022). MMS alkylates mRNA, which creates bulky mRNA adducts that cause ribosome stalling by interfering with aminoacyl-tRNA accommodation (Thomas *et al*, 2020; Yan & Zaher, 2021). All these conditions induced ubiquitination of RPS27A/eS31 in both WT and *USP16* KO cells (Fig 1B-D). This indicates that ubiquitination of RPS27A/eS31 is a general response to drug-induced ribosome stalling, irrespective of the mechanism by which stalling is achieved.

Next, we wished to explore which physiological conditions can induce RPS27A/eS31 ubiquitination. It is known that amino acid starvation can cause stalling of ribosomes by reducing the levels of the respective aminoacyl-tRNAs (Darnell *et al*., 2018; Worpenberg *et al*, 2025; Zhou *et al*., 2025). We therefore cultivated WT and *USP16* KO cells in the absence of arginine, lysine or both. Immunoblot analysis revealed that in both cell lines, levels of ubiquitinated RPS27A/eS31 increased in the absence of amino acids (Fig 1E-G).

Altogether, these results indicate that ubiquitination of RPS27A/eS31 is induced by a variety of conditions that cause ribosome stalling. Importantly, also physiological shortage of amino acids as occurring during amino acid starvation can trigger RPS27A/eS31 ubiquitination.

Ubiquitination of RPS27A/eS31 depends on RNF25 and GCN1

Recent studies have identified RNF25 as a candidate E3 ubiquitin ligase responsible for the ubiquitination of the RPS27A/eS31 (Gurzeler *et al*., 2023; Oltion *et al*., 2023). To investigate whether the elevated levels of RPS27A/eS31 ubiquitination in *USP16* KO cells induced by translation inhibitors or amino acid starvation can indeed be attributed to the action of RNF25, we generated *USP16/RNF25* double knockout (DKO) cell lines from parental HeLa *USP16* KO cells using CRISPR/Cas9. The *RNF25* deletion was confirmed by both genotyping and immunoblotting (Fig S2A, S2B). In addition, we validated inactivation of the RNF25 pathway by treating cells with SRI-41315, which traps eRF1 on ribosomes and leads to its RNF25-dependent ubiquitination and subsequent degradation (Oltion *et al*., 2023). As expected, knockout of *RNF25* prevented the degradation of eRF1 (Fig S2B), confirming that our knockout was functional. Compared to the parental *USP16* KO cells, *USP16/RNF25* DKO cells failed to induce ubiquitination of RPS27A/eS31 upon amino acid starvation (Fig 2A, 2B). Thus, the rise in the levels of ubiquitinated RPS27A/eS31 upon ribosome stalling in response to amino acid starvation depends on RNF25.

Several proteins have been described as sensors of stalled/collided ribosomes: ZNF598, GCN1, EDF1 and ZAKα (Huso *et al*., 2026; Juszkiewicz *et al*., 2018; Pochopien *et al*., 2021; Sinha *et al*., 2020) (Fig 3A). We have previously demonstrated that ZNF598 is not involved in RPS27A/eS31 ubiquitination (Montellese *et al*., 2020), although it mediates the ubiquitination of other ribosomal proteins in response to ribosome stalling (Juszkiewicz & Hegde, 2017; Sundaramoorthy *et al*., 2017). In contrast, the RNF25 pathway is activated through the recognition of collided ribosomes by GCN1 (Gurzeler *et al*., 2023; Oltion *et al*., 2023). To define whether only GCN1, or also EDF1 and ZAKα, are involved in ubiquitination of RPS27A, we depleted these factors in *USP16* KO cells using siPOOLs (Fig 3B-D), followed by ternatin treatment to induce ribosome stalling and to activate the RNF25 pathway. The knockdown of GCN1 led to reduced levels of ubiquitinated RPS27A/eS31 under control conditions, and no increase in ubiquitination upon ribosome stalling, as expected. In contrast, depletion of EDF1 and ZAKα had no effect on the levels of ubiquitinated RPS27A/eS31 compared to control conditions, in the presence or absence of ternatin (Fig 3B-D). These data indicate that GCN1 is required for RNF25-dependent RPS27A/eS31 ubiquitination, whereas EDF1 and ZAKα are not.

To test whether GCN1 is also involved in starvation-induced RPS27A/eS31 ubiquitination, we generated HeLa *USP16/GCN1* DKO cell lines (Fig S2C, S2D). As expected, compared to the parental *USP16* KO cells, RPS27A/eS31 modification was impaired in the *USP16/GCN1* DKO. Importantly, amino acid starvation failed to induce RPS27A/eS31 modification in the absence of GCN1 (Fig 3E, 3F). This confirms that GCN1 is required for RPS27A/eS31 ubiquitination in response to decreased availability of amino acids.

Taken together, our results indicate that the ubiquitination of RPS27A/eS31 depends on the recognition of collided ribosomes by GCN1 and recruitment of RNF25. Other collision sensors, such as EDF1 and ZAKα, seem dispensable for RPS27A/eS31 ubiquitination.

Ubiquitination of RPS27A/eS31 is needed to support translation

To understand how inactivation of the RNF25 pathway influences translation, we analysed polysome profiles of HeLa WT cells upon knockdown of RNF25 (Fig 4A-C). Interestingly, in the absence of RNF25, cells displayed slightly decreased levels of 80S monosomes, but the amount of polysomes was similar to control cells, resulting in an increased polysome/monosome (P/M) ratio. This might indicate that RNF25 is needed to support clearance of transiently stalled ribosomes already under optimal growth conditions, likely because transient stalling events of ribosomes occur naturally during unperturbed translation.

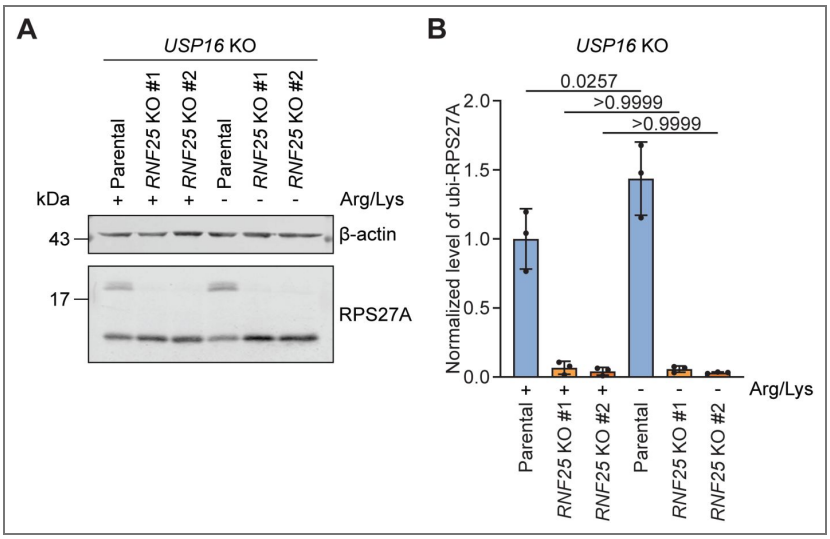

Figure 2. Ubiquitination of RPS27A/eS31 depends on RNF25.

(A) Analysis of RPS27A/eS31 ubiquitination in HeLa *USP16* KO cells upon deletion of *RNF25*. The *RNF25* gene was deleted from *USP16* KO cells by CRISPR/Cas9. The parental *USP16* KO cells and two clones of *USP16*/*RNF25* double knockout (DKO) cells were cultivated for 6 h in the presence (+) or absence (-) of arginine and lysine. Cell extracts were analysed by immunoblotting with the indicated antibodies. **(B)** Quantification of levels of ubiquitinated RPS27A/eS31 in cell lysates from (A), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to samples from non-starved parental *USP16* KO cells (N = 3, mean ± SD, one-way ANOVA and post hoc Tukey's test, p-values are indicated).

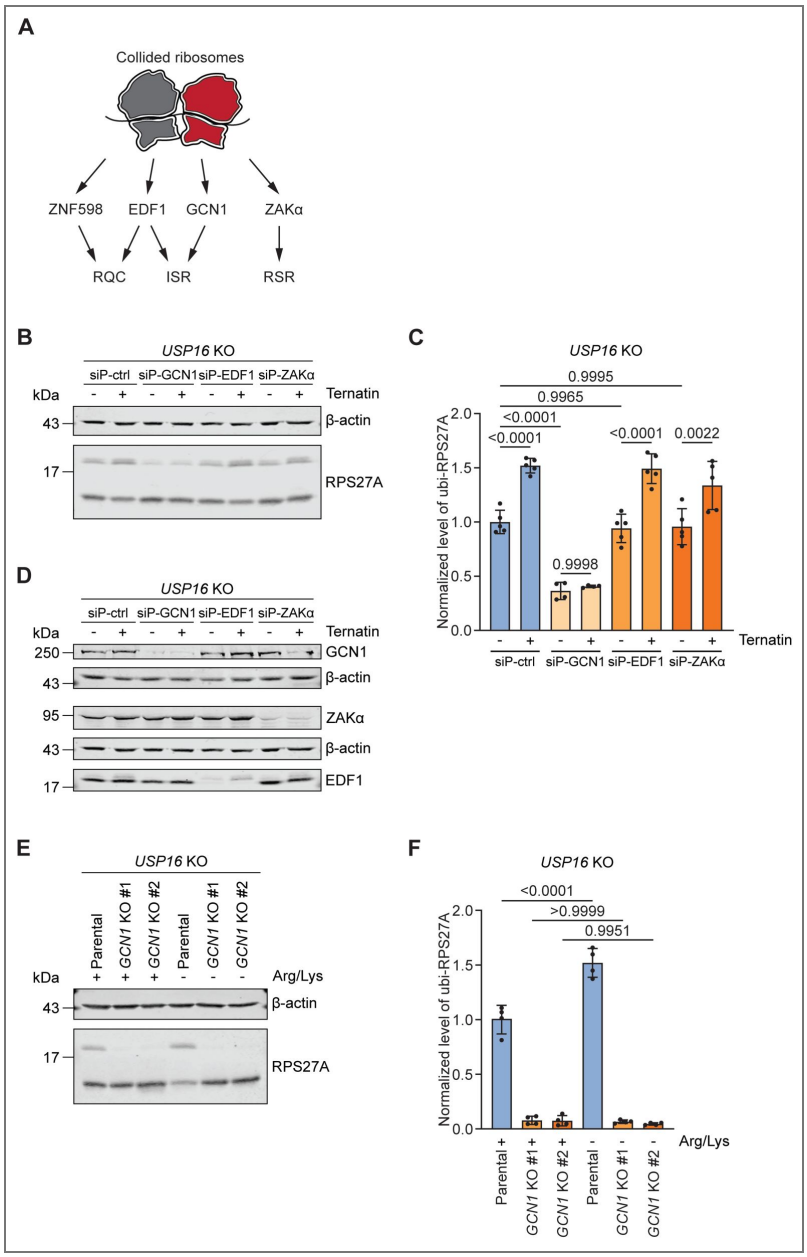

Figure 3. Ubiquitination of RPS27A/eS31 depends on GCN1 but not on EDF1 or ZAKα.

(A) Schematic depiction of various sensors that recognize collided ribosomes in three major quality control pathways: ribosome-associated quality control (RQC), integrated stress response (ISR) and ribotoxic stress response (RSR). **(B)** Analysis of RPS27A/eS31 ubiquitination in HeLa *USP16* KO cells upon depletion of GCN1, EDF1 or ZAKα. *USP16* KO cells were treated for two times 48 h with siPOOLs (control or targeting the mRNAs of GCN1, EDF1 or ZAKα). Prior to harvesting, cells were cultivated for 4 h in the absence (-) or presence (+) of 50 nM ternatin. Cell extracts were analysed by immunoblotting with the indicated antibodies. **(C)** Quantification of levels of ubiquitinated RPS27A/eS31 in cell lysates from (B), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to samples from untreated control cells (N ≥ 4, mean ± SD, one-way ANOVA and post hoc Tukey's test, p-values are indicated). **(D)** Validation of knockdown experiments from (B). The efficiency of downregulation was analysed by immunoblotting of cell extracts with the indicated antibodies. **(E)** Analysis of RPS27A/eS31 ubiquitination in HeLa *USP16* KO cells upon deletion of *GCN1*. The *GCN1* gene was deleted from *USP16* KO cells by CRISPR/Cas9. The parental *USP16* KO cells and two clones of *USP16*/*GCN1* DKO cells were cultivated for 6 h in the presence (+) or absence (-) of arginine and lysine. Cell extracts were analysed by immunoblotting with the indicated antibodies. **(F)** Quantification of levels of ubiquitinated RPS27A/eS31 in cell lysates from (E), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to samples from non-starved parental *USP16* KO cells (N = 4, mean ± SD, one-way ANOVA and post hoc Tukey's test, p-values are indicated).

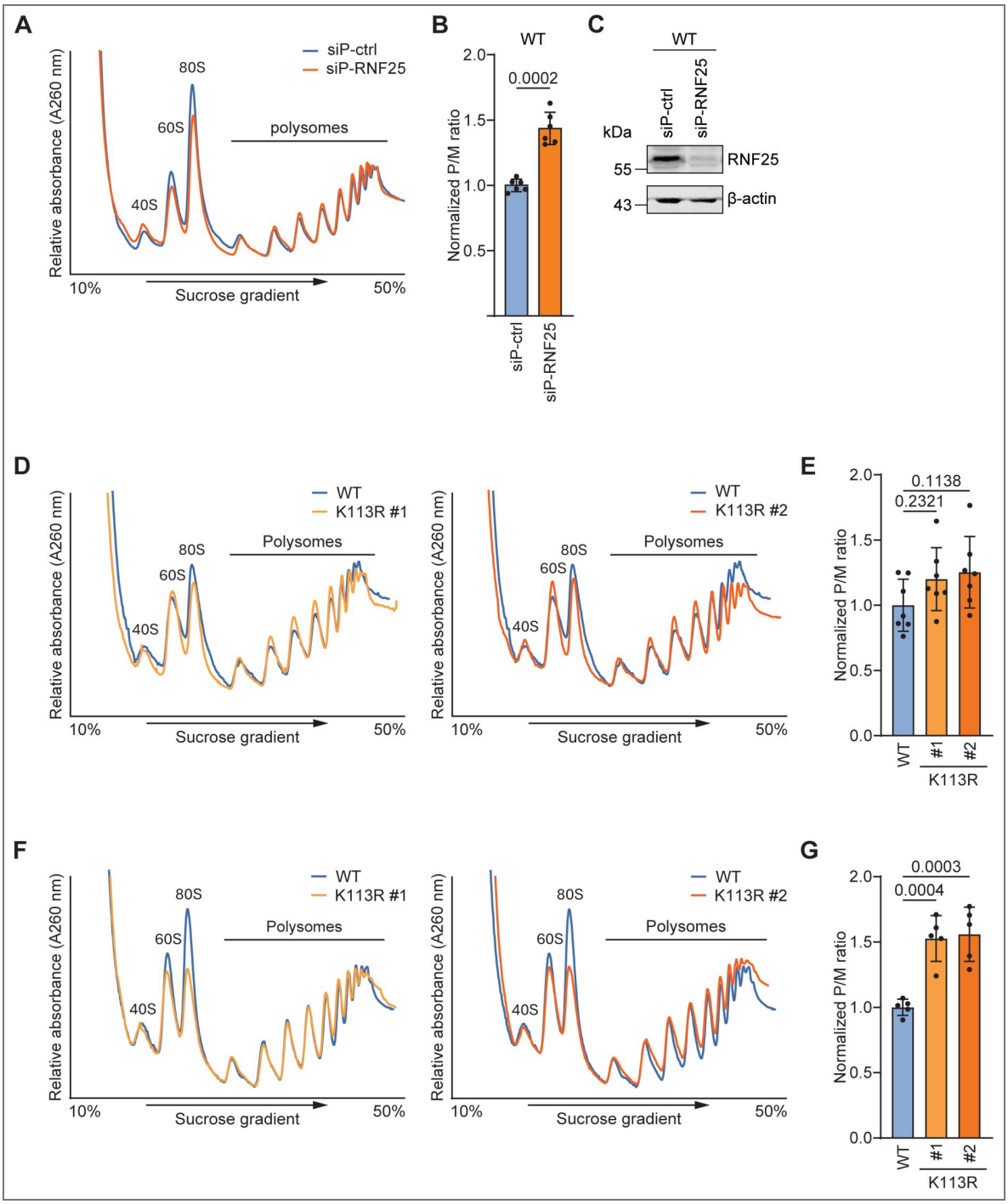

Figure 4. Ubiquitination of RPS27A/eS31 is needed to support translation.

(A) Analysis of polysome profiles upon RNF25 knockdown. RNF25 was depleted from HeLa cells by treatment with siPOOLs for 72 h. Cell extracts were separated by sedimentation in 10%-50% sucrose density gradients and profiles were visualized at 260 nm. **(B)** Quantification of polysome/monosome (P/M) area ratios in profiles from (A), normalized to control profiles (N = 4, mean ± SD, unpaired two-tailed Student's t-test, p-value = 0.0002). **(C)** Validation of knockdown experiments from (A). The efficiency of downregulation was analysed by immunoblotting of cell extracts with the indicated antibodies. **(D)** Analysis of polysome profiles upon inactivation of the RNF25 pathway. Cell extracts from parental HeLa WT cells or gene-edited *RPS27A* K113R cells were separated by sedimentation in 10%-50% sucrose density gradients and profiles were visualized at 260 nm. **(E)** Quantification of polysome/monosome (P/M) area ratios in profiles from (D), normalized to WT profiles (N = 7, mean ± SD, one-way ANOVA and post hoc Dunnett's test, p-values are indicated). **(F)** Analysis of polysome profiles of HeLa WT and RPS27A K113R cells upon ISRIB treatment. Parental HeLa WT cells or gene-edited *RPS27A* K113R cells were treated with 1.1 µM ISRIB for 6 h. Cell extracts were separated by sedimentation in 10%-50% sucrose density gradients and profiles were visualized at 260 nm. **(G)** Quantification of polysome/monosome (P/M) area ratios in profiles from (F), normalized to WT profiles (N = 5, mean ± SD, one-way ANOVA and post hoc Dunnett's test, p-values are indicated).

To examine the functional importance of the RNF25-mediated ubiquitination of RPS27A/eS31 in this regard, we utilized genome-edited HeLa cells harbouring a K113R substitution in RPS27A/eS31, which prevents its RNF25-dependent ubiquitination (Fig S3A, S3B). These cells are therefore deficient in the RNF25 pathway, evident by the lack of eRF1 degradation upon treatment of cells with SRI-41315 (Fig S3B). Curiously though, there was no significant difference in the P/M ratio in lysates of K113R and WT cells growing under optimal conditions, although the P/M ratio was slightly elevated (Fig 4D, 4E). We reasoned that the difference to the RNF25 knockdown experiments might be explained by the possibility that upon long-term inactivation of the RNF25 pathway, as in the K113R mutant cells, other pathways take over to resolve stalled ribosomes. Intriguingly, when cultivating K113R cells in the presence of ISRIB, which activates translation by reducing the inhibitory effect of phospho-eIF2α and thereby antagonising the ISR, we could see a substantial increase in the P/M ratio compared to WT cells (Fig 4F, 4G). These data further support the notion that activation of the RNF25 pathway is needed not only to deal with collisions caused by external stimuli, but also to resolve transient ribosome collisions occurring naturally under optimal growth conditions.

Level of ubiquitinated RPS27A/eS31 increases in the absence of GCN2 and DRG2/RWDD1

Our data thus far indicate that RNF25-dependent ubiquitination of RPS27A/eS31 is dependent on the presence of GCN1 and is induced upon amino acid starvation (Fig 1E-G and 3B-F). GCN1 is not only involved in the RNF25 pathway, but it is also a part of the ISR, which can be induced by multiple triggers including amino acid starvation. In the ISR pathway, GCN1 recognizes collided ribosomes and recruits GCN2, which is subsequently activated and autophosphorylated (Darnell *et al*., 2018; Hinnebusch, 1994, 2005; Marton *et al*., 1993; Masson, 2019; Zhou *et al*., 2025). Thus, both GCN2 and RNF25 rely on the recognition of collided ribosomes by GCN1 for their activation. There are at least two possible scenarios to reconcile the functional relationship between these factors: either GCN2 and RNF25 belong to the same pathway, or they are parts of two independent parallel pathways, both depending on GCN1.

To examine whether RNF25 is involved in the activation of the ISR, we compared levels of phospho-GCN2 in WT and K113R cells upon amino acid starvation (Fig S4A, S4B). Immunoblot analysis revealed comparable levels of phospho-GCN2 between the different cell lines, indicating that ubiquitination of RPS27A/eS31 by RNF25 is not required for the activation of GCN2 as part of the ISR and that the two pathways diverge downstream of GCN1.

To test the idea that the ISR and RNF25 pathways co-exist as two parallel pathways, we generated *USP16/GCN2* DKO cell lines from parental HeLa *USP16* KO cells (Fig S4C, S4D). Interestingly, the levels of ubiquitinated RPS27A/eS31 increased in the absence of GCN2 already in non-starved conditions (Fig 5A, 5B), and there was no further increase in the ubiquitination levels upon amino acid starvation. This indicates that in the absence of GCN2, the RNF25 pathway is already fully activated by naturally occurring translational stalling events and supports the hypothesis that RNF25 and GCN2 represent two independent pathways downstream of GCN1, guarding cells against ribosome collisions.

Both RNF25 and GCN2 possess RWD domains that can bind the RWDBD domain of GCN1, raising the possibility that RNF25 and GCN2 compete for GCN1 binding via their RWD domains. Comparative analysis of the respective RWD domains with ClustalW as well as AlphaFold prediction of interactions between these domains and the RWDBD domain of GCN1 suggest that binding occurs using a similar interaction interface (Fig 5C, 5D). In both cases, polar interactions involved GCN1 residues R2312, R2317 and K2354, with R2312 forming contacts with the positionally conserved residues E30 of RNF25 or D37 of GCN2 (Fig 5C, 5D). This model agrees with previous observations describing that a R2312A substitution in GCN1 prevents binding and activation of GCN2 (Sattlegger & Hinnebusch, 2000; Zhou *et al*., 2025). To test the idea that the RWD domains of RNF25 and GCN2 compete for GCN1 binding, we overexpressed the wild-type RWD domain of GCN2 in HeLa *USP16* KO cells, as well as a mutant version harbouring D37K, E125K and E136K substitutions (3K mutant), which potentially has a hampered interaction with

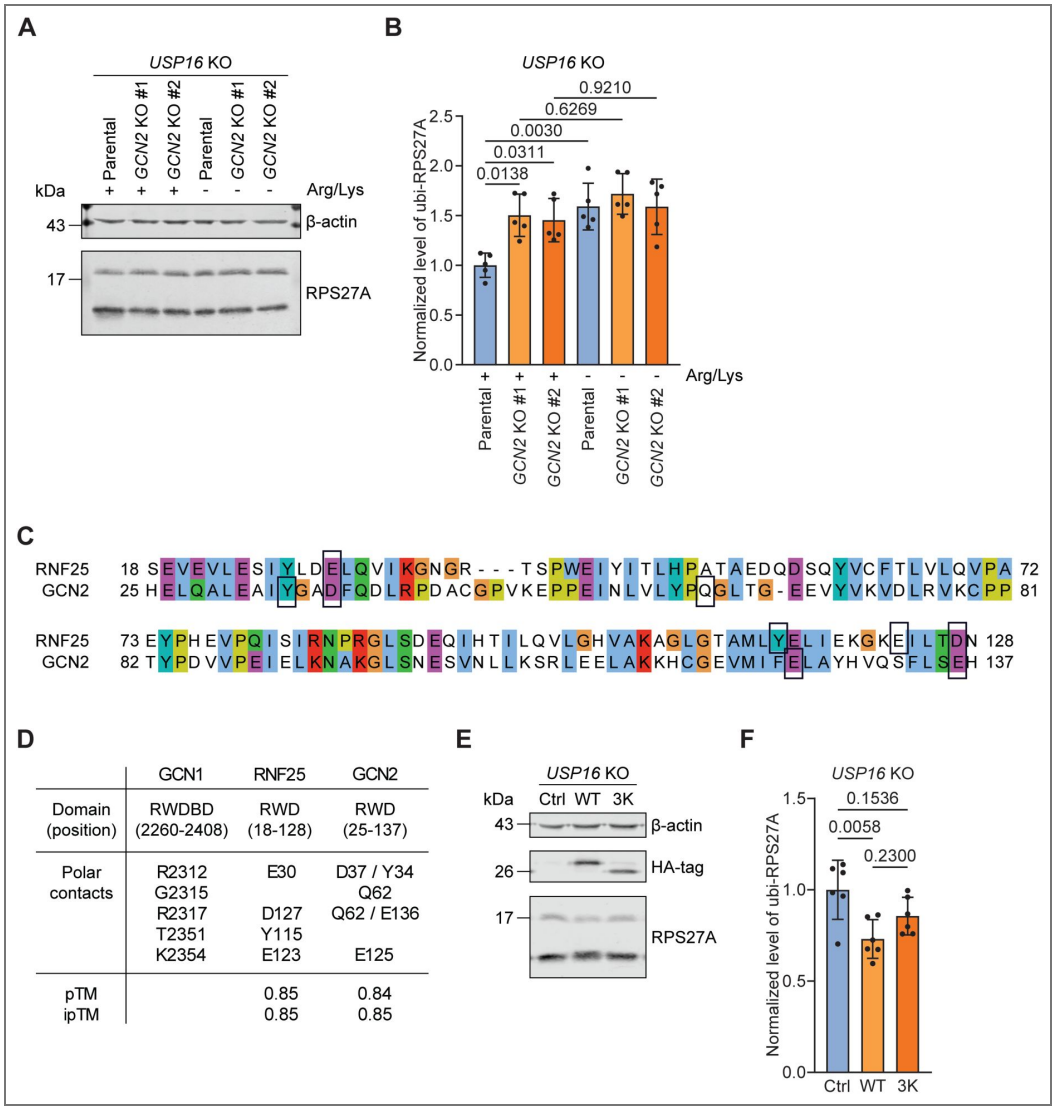

Figure 5. Level of ubiquitinated RPS27A/eS31 increases in the absence of GCN2

(A) Analysis of RPS27A/eS31 ubiquitination in HeLa *USP16* KO cells upon deletion of *GCN2*. The *GCN2* gene was deleted from *USP16* KO cells by CRISPR/Cas9. The parental *USP16* KO cells and two clones of *USP16*/GCN2 double knockout (DKO) cells were cultivated for 6 h in the presence (+) or absence (-) of arginine and lysine. Cell extracts were analysed by immunoblotting with the indicated antibodies. **(B)** Quantification of levels of ubiquitinated RPS27A/eS31 in cell lysates from (A), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to samples from non-starved parental *USP16* KO cells (N = 5, mean ± SD, one-way ANOVA and post hoc Tukey's test, p-values are indicated). **(C)** Sequence alignment of RWD domains of RNF25 (aa residues 18-128) and GCN2 (aa residues 25-137). Sequences were visualised in Jalview and aligned with Clustal W. Residues predicted by AlphaFold 3 web server to interact with RWDBD domain of GCN1 are indicated with rectangles. Residues are colored according to the Clustal X color scheme: blue – hydrophobic, red – positive charge, magenta – negative charge, green – polar, orange – glycine, yellow – proline, cyan – aromatic. **(D)** Prediction of interaction between RWDBD domain of GCN1 and RWD domains of RNF25 and GCN1 using AlphaFold 3. Protein domains as well as positions of respective residues used for the modelling are indicated. Respective pTM and ipTM scores show high-quality predictions (> 0.8) for both the overall protein structure (pTM) and the protein-protein complex (ipTM). Indicated polar contacts were analysed with PyMol. **(E)** Analysis of RPS27A/eS31 ubiquitination in HeLa *USP16* KO cells upon transient overexpression of the indicated GCN2 fragments containing an N-terminally StStHA-tagged RWD domain (aa 1-145). The effect of WT and 3K mutant (D37K, E125K and E136K substitutions) RWD domains was compared to control conditions (transfection of empty vector). Cell extracts were analysed by immunoblotting with the indicated antibodies. **(F)** Quantification of levels of ubiquitinated RPS27A/eS31 in cell lysates from (E), expressed as the ratio between ubi-RPS27A/eS31 and total RPS27A/eS31 (ubi-RPS27A/eS31 + unmodified RPS27A/eS31) and normalized to control samples (N = 6, mean ± SD, one-way ANOVA and post hoc Tukey's test, p-values are indicated).

GCN1. The levels of ubiquitinated RPS27A/eS31 decreased about 1.4-fold upon expression of wild-type RWD domain of GCN2 (Fig 5E, 5F), supporting the idea that this domain competes with RNF25 for GCN1 binding. Cells expressing the 3K mutant had intermediate levels of ubiquitinated RPS27A/eS31, indicating that the interface mutations weaken GCN1 interaction. Altogether, these data support the idea that the RWD domains of RNF25 and GCN2 compete for GCN1 binding.

It is important to note that two further proteins, RWDD1 and Impact, contain RWD domains capable of GCN1 binding (Ishikawa *et al*, 2013; Pereira *et al*, 2005; Pochopien *et al*., 2021; Waller *et al*, 2012; Wout *et al*, 2009). Yeast homologs of RWDD1 and its cofactor DRG2, with the respective names Gir2 and Rbg2, have been previously identified in a cryo-EM model of collided ribosomes, where both proteins reside in the A-site factor binding region of the leading ribosome, and Gir2 mediates an interaction between the Rbg2/Gir2 complex and Gcn1 (Pochopien *et al*., 2021). To test whether the human DRG2/RWDD1 complex represents another pathway competing with RNF25, we generated *USP16/DRG2* DKO cells using parental HeLa *USP16* KO (Fig S5A). As expected (Ishikawa *et al*, 2009), these cells exhibited decreased levels of RWDD1 upon depletion of its cofactor DRG2, reflecting the degradation of excess RWDD1 protein when formation of the DRG2/RWDD1 complex is prevented (Fig S5B, S5C). In turn, the levels of ubiquitinated RPS27A/eS31 increased in the absence of DRG2 (Fig S5B, S5D), which might be caused by reduced competition between RWDD1 and RNF25, and a consequent inability to resolve ribosome stalling through the DRG2 pathway. Altogether, these results suggest that GCN1-dependent quality control of protein synthesis has evolved as a multifaceted process involving several parallel downstream pathways.

Discussion

Recent studies have identified a novel translation-dependent quality control pathway involving the E3 ligase RNF25, which ubiquitinates RPS27A/eS31 (Gurzeler *et al*., 2023; Oltion *et al*., 2023). Here, we used ubiquitination levels of RPS27A/eS31 as an indicator of RNF25 activation to systematically investigate the triggers and mechanism of this pathway.

Our data indicate that ubiquitination of RPS27A/eS31 is induced by a variety of conditions that cause ribosome stalling. First, we established that besides ternatin, the presence of other translation inhibitors, such as emetine, didemnin B, anisomycin or MMS, leads to the activation of RNF25. Interestingly, these drugs have different modes of action, not always leading to trapped translation factors in the ribosomal A-site. Didemnin B acts similarly to ternatin by locking eEF1A in the A-site of the ribosome (Juette *et al*., 2022). Emetine binds to the E-site of the 40S subunit and prevents translocation, leaving eEF2 bound to the ribosomal A-site (Wong *et al*., 2014; Zhou *et al*., 2025). Anisomycin binds at the peptidyl transferase centre of the 60S subunit and inhibits peptide bond formation. In the presence of anisomycin, incoming aminoacyl-tRNA can still undergo the codon-anticodon pairing step, but the correct placement of the aminoacyl-3'-CAA end of the tRNA is abolished. This stalls ribosomes in the pre-peptide bond formation conformation with a partially accommodated tRNA (Garreau de Loubresse *et al*., 2014; Wu *et al*, 2019a). MMS is a nucleic acid damaging reagent that induces RNA methylation. Presence of bulky methyl groups on mRNAs was suggested to prevent correct aminoacyl-tRNA accommodation, leaving ribosomes stalled with an empty A-site (Stoneley *et al*., 2022; Thomas *et al*., 2020; Yan & Zaher, 2021). Importantly, we could also identify amino acid starvation as a physiological trigger that activates the RNF25 pathway, leading to the ubiquitination of RPS27A/eS31. Under starvation conditions, ribosome stalling is caused by the lack of aminoacyl-tRNA. However, uncharged tRNA might still bind to the A-site of the ribosome and base-pair with cognate codons with the help of the GCN2 kinase, which contributes to the activation of GCN2 on the ribosome (Zhou *et al*., 2025). Thus, our analyses demonstrate that a variety of triggers leading to diverse ribosomal states can activate RNF25, suggesting that activation of this pathway does not *per se* require the presence of trapped protein factors in the ribosomal A-site but is a more general response to ribosome collisions.

Ribosomal collisions are recognized by several sensors, namely EDF1, ZNF598, GCN1 and ZAKα. We have previously excluded a role for ZNF598 in RPS27A/eS31 ubiquitination (Montellese *et al.*, 2020). Here, we investigated whether ubiquitination of RPS27A/eS31 depends on the presence of EDF1, GCN1 or ZAKα. Two of these sensors – EDF1 and GCN1 – are involved in the activation of the ISR pathway (Kim *et al.*, 2024; Marton *et al.*, 1993; Pochopien *et al.*, 2021), while ZAKα is involved in the RSR pathway (Huso *et al.*, 2026; Sinha *et al.*, 2024; Vind *et al.*, 2020). Our analysis demonstrated that ubiquitination of RPS27A/eS31 does not depend on the presence of EDF1 or ZAKα, but on GCN1, in agreement with earlier observations (Gurzeler *et al.*, 2023; Oltion *et al.*, 2023). A previous structural study of collided disomes in yeast has identified the C-terminal part of Gcn1 (with its RWD domain) in vicinity of yeast Rps31/eS31 (Pochopien *et al.*, 2021), suggesting that also on mammalian disomes, GCN1 would be ideally positioned to guide RNF25 to RPS27A/eS31 for its ubiquitination.

Intriguingly, our analysis showed that the RNF25 pathway is needed to support translation already under optimal growth conditions, as inactivation of this pathway leads to an increased polysome/monosome ratio, hinting at the general presence of low levels of unresolved ribosome collisions. This indicates that the RNF25 pathway is active to resolve collisions caused not only by translational stress, but also transient events naturally occurring during active translation. Interestingly, a recent study using *in situ* cryo-electron tomography indeed detected GCN1 on 2% of collided ribosomes in untreated cells and at 6% of disomes upon anisomycin treatment, whereas it was excluded from ribosomes in compact and persistent collisions (Fedry *et al*, 2024). It has also been reported that transient ribosome collisions caused by nonoptimal codons or in 3' UTR regions are recognized by GCN1 (Muller *et al*, 2023). Moreover, we previously showed that G418, a drug that induces stop codon readthrough, induces ubiquitination of RPS27A/eS31 (Montellese *et al.*, 2020), suggesting that the RNF25 pathway is involved in the resolution of collisions in the 3'UTR of mRNAs. Since activity of the RNF25 pathway depends on the presence of GCN1, it is possible that GCN1 and RNF25 act in tandem to recognize "loose" collisions produced by transient stalling events.

GCN1 is an integral part of the ISR pathway, where it participates in the activation of GCN2 on ribosomes upon amino acid starvation. Active GCN2 inhibits translation initiation by phosphorylating the α subunit of eIF2. Surprisingly, we found that the level of ubiquitinated RPS27A/eS31 increases in the absence of GCN2 already in non-starved conditions, hinting at a competition between GCN2 and RNF25. Both proteins possess structurally similar RWD domains that can bind the RWDBD domain of GCN1, suggestive of a mechanism where competition of RNF25 and GCN2 is at least partially mediated by these domains. In support of this idea, we observed that overexpression of the RWD domain of GCN2 leads to a decreased level of ubiquitinated RPS27A/eS31. While this manuscript was in preparation, two other studies described competitive relations between RNF25 and GCN2, where RNF25 protects cells from the GCN2-dependent activation of ISR upon RNA damage (Seidel *et al*, 2026; Zhao *et al*, 2026). In turn, our data support a broader model according to which such competition occurs not only in response to RNA damage (MMS treatment, Fig 1B-D), but also during amino acid starvation as well as upon transient stalling in non-starved conditions (Fig 5A, 5B).

Further extending the complexity of the regulatory network, our data indicates that the DRG2/RWDD1 complex represents another GCN1-dependent pathway that competes with RNF25. To date, little is known about how the DRG2/RWDD1 pathway intersects with other RQC factors. A recent cryo-EM structure of yeast GCN1-bound disomes has visualised the yeast Rbg2/Gir2 complex (homologs of DRG2/RWDD1) in the A-site factor binding region of the ribosome, where Rbg2 directly interacts with the A-site tRNA and Gir2 mediates interaction of the complex with the RWDBD domain of Gcn1 (Pochopien *et al.*, 2021). Based on this structure it has been suggested that Rbg2 stabilizes the accommodated A-site tRNA and promotes efficient translation upon ribosome pausing (Pochopien *et al.*, 2021). Moreover, binding of the Rbg2/Gir2 complex has been shown to inhibit ISR activation by preventing the recruitment of Gcn2 (Ishikawa *et al.*, 2013). Therefore, it is possible that in human cells the RNF25 and DRG2/RWDD1 pathways have overlapping functions. RNF25 may act as a general sensor of stalled ribosomes, recognizing

ribosomes with empty, t-RNA and/or factor occupied A-sites. DRG2/RWDD1, in contrast, seems to specifically recognises stalled ribosomes that lack a protein factor but have tRNA bound in the A-site. However, more work must be done to unravel the relationship between these two pathways.

Collectively, our work revealed that (i) the RNF25 pathway can be activated by various triggers that affect A-site occupancy in distinct ways, (ii) loss of RNF25 increases the polysome/monosome ratio in untreated cells, suggesting a role in clearance of collisions that occur naturally during translation, (iii) GCN1 serves as a central factor of a regulatory network at which GCN2 and DRG2/RWDD1 compete with the RNF25 pathway. Based on these observations, we propose a model in which the RNF25 pathway is activated as a general response to a variety of ribosome collisions (low doses of translation inhibitors, RNA damage, amino acid starvation, transient collision events) (Fig 6). For the activation of this pathway, such ribosome collisions are first recognized by GCN1, which subsequently recruits RNF25 via its RWD domain. As a result, RNF25 ubiquitinates RPS27A/eS31, which in turn induces the resolution of stalled ribosomes. Initially, RNF25, which is likely only present in very low amounts (23'300 molecules per HeLa cell (Itzhak *et al*, 2016)), is recruited to resolve such transient stalling events. As a parallel pathway, DRG2/RWDD1 recognizes specific stalling events where ribosomes slow down and have a vacant A-site factor binding region. Once collisions increase above a certain threshold or persist, GCN2 is activated, likely with overall increasing amount of GCN1 on collided ribosomes, leading to the activation of the IRS pathway and inhibition of translation.

Materials and methods

Cell lines, culturing conditions, and treatments

HeLa cells were cultured in DMEM supplemented with 10% FCS and 100 µg/ml penicillin/streptomycin at 37°C and 5% CO_2. HeLa K WT cells and HeLa K *USP16* KO were described previously (Montellese *et al*., 2020). Where indicated, cells were treated with following compounds: anisomycin (Sigma, A9789), emetine (Sigma, E2375), didemnin B (Sigma, SML4022), ternatin (Santa Cruz, sc-391653), methyl methanesulfonate (Sigma, 129925), SRI-41315 (Sigma, SML3312), ISRIB (Sigma, SML0843).

In amino acid starvation experiments, cells were grown in DMEM for SILAC (Thermo Scientific, 88364) supplemented with 10% dialyzed FCS (Gibco, A3382001), 100 µg/ml penicillin/streptomycin, 84 µg/ml arginine and 146 µg/ml lysine. To induce amino acid starvation, arginine and/or lysine were omitted from the media where indicated.

RNA interference

HeLa cells growing on either 6-well or 10 cm plates were transfected with 5 nM siPOOLs (siTOOLs, (Hannus *et al*, 2014)) in Opti-MEM (Gibco, 31985047) using INTERFERin (Polyplus, 101000028). Medium was exchanged on the next day.

Molecular cloning and transfection of RWD domain-encoding constructs

DNA fragments encoding for the RWD domain of human GCN2 (aa residues 1-147, WT and 3K mutant) were ordered from TWIST Bioscience and inserted into pcDNA/FRT/TO vector (Invitrogen) with N-terminal StStHA-tag using KpnI and XhoI cloning sites.

HeLa K *USP16* KO cells growing on 6-well plates were transfected with the RWD domain encoding vectors using the jetPrime reagent (Sartorius, 101000046). Media was exchanged 6 h post-transfection, and cells were harvested 48 h post-transfection.

Generation of knockout cell lines

Double knockout (DKO) HeLa cell lines (*USP16/RNF25* DKO, *USP16/GCN1* DKO, *USP16/GCN2* DKO and *USP16/DRG2* DKO) were generated using a CRISPR-Cas9 genome editing system. The following gRNA sequences were used in this study: RNF25 exon 3: 5' AAAGTGATGTAGATCTCCCA 3' (Oltion *et*

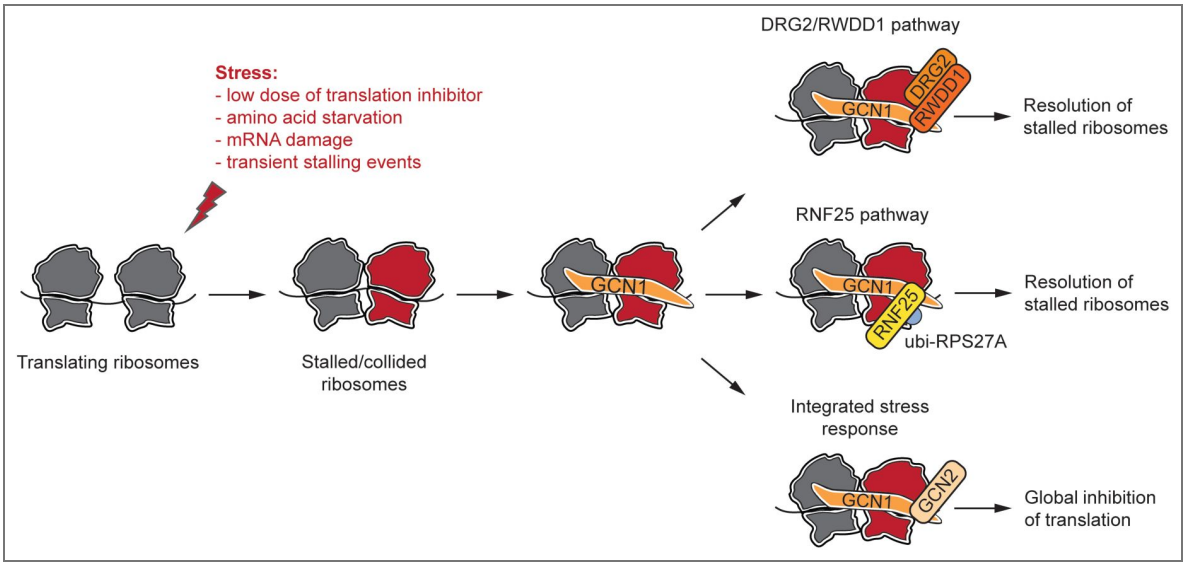

Figure 6. **Model for the activation of the RNF25 pathway.**

Upon conditions that induce ribosome stalling (such as low doses of translation inhibitors, mRNA damage or amino acid starvation) as well as upon natural transient stalling events, collided ribosomes are recognized by GCN1. Following GCN1 binding, RNF25 is recruited onto ribosomes through the interaction between its RWD domain and the RWDBD domain of GCN1, resulting in the ubiquitination of RPS27A/eS31 and subsequent resolution of stalled ribosomes. Alternatively, the DRG2/RWDD1 complex can also interact with GCN1 and engage in resolution of stalled ribosomes that have no protein factor in the A-site factor binding region, but a tRNA bound in the A-site. With an increased number of collisions or more persistent collisions, GCN1 recruits GCN2 through interaction with its RWD domain, which induces activation of the ISR pathway and inhibition of translation.

al., 2023) GCN1 exon 14: 5' AAACCTCCACATCTGCGGTG 3' (Doench *et al*, 2016) GCN2 exon 3: 5' ACTGGCCAAGAAACACTGTG 3' (Doench *et al*., 2016) DRG2 exon 1: 5' GCTCGGACACAGAAGAACAA 3' (Doench *et al*., 2016) gRNA sequences were inserted into the BsaI site of the pC2P vector containing hCas9 and a puromycin resistance cassette (Welte *et al*, 2019). HeLa K *USP16* KO cells growing on 6-well plates were transfected with the resulting vector using the jetPrime reagent (Sartorius, 101000046). The next day, cells were re-seeded onto 10 cm plates. After 24 h, and selection was started with 1 µg/ml of puromycin for 2 days. After selection, individual clones were expanded, and the knockouts were verified by Sanger sequencing and immunoblotting. Sequencing results were decomposed either manually or using TIDE web service (Brinkman *et al*, 2014).

Prime editing

HeLa K cells harbouring the base substitutions at the endogenous RPS27A/eS31 loci for expression of the K113R mutant were generated using prime editing (Doman *et al*, 2022). Specifically, the sequence 5'-GAATGGC**AAA**ATT-3' located at position 55235436-55235448 on the positive strand of chromosome 2 was substituted to a sequence 5'-GAACGGC**CGA**ATT-3'. This resulted in the substitution of AAA®CGA (Lys®Arg, bold) and introduction of a silent EagI digestion site (underlined). epegRNA targeting RPS27A/eS31 was designed using PrimeDesign tool (Hsu *et al*, 2021), and consisted of the following parts:

Spacer: 5' CAGAAGGGCACTCTCGACGA 3'

Scaffold: 5' CTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG 3'

Extension (41 nt RTT and 12 nt PBS): 5' GCTTTTCAGGTGGATGAGAACGGCCGAATTAGTCGGCTTCGTC GAGAGTGCCC 3'

Spacer, scaffold and extension of epegRNA were assembled using Golden Gate cloning and inserted into the BsaI site of the pU6-tevopreq1-GG-acceptor vector, which was a gift from David Liu (Addgene plasmid # 174038) (Nelson *et al*, 2022). PEmax prime editor was expressed from the pCMV-PEmax vector, encoding for SpCas9 and MMLV-RT (a gift from David Liu, Addgene plasmid # 174820) (Chen *et al*, 2021). pIRESpuro encoding for a puromycin resistance cassette was used for selection of cells with puromycin.

HeLa K WT cells growing on 6-well plates were transfected with pIRESpuro, PEmax and epegRNA vectors at a ratio 0.1 ug : 1 ug : 1.2 ug using jetPrime. Next day, cells were re-seeded onto 10 cm plates. After 24 h, cells were selected with 1 µg/ml puromycin for 2 days. After selection, individual clones were picked and expanded. To verify the edits, genomic DNA was extracted with phenol/chloroform/IAA (Applichem, A0889.0500) and analyzed by digestion of PCR products with EagI and Sanger sequencing.

Immunoblot analysis and antibodies

Cells growing on 6-well plates were washed once with ice-cold 1x PBS and collected by scraping in 120 µl of lysis buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM N-ethylmaleimide, 1 ug/ml pepstatin, 10 ug/ml leupeptin, 10 ug/ml aprotinin). In case of analysis of phosphorylated proteins, lysis buffer was additionally supplemented with 0.5 mM NaF and 0.1 mM $NaVO_4$. Cells were incubated on ice for 5 min with occasional vortexing, and the resulting lysates were cleared by centrifugation (16,000 x g, 10 min, 4°C). Protein concentration was measured with the Bradford method using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, 5000006), and equal amounts of protein were supplemented with SDS loading buffer. Samples were denatured for 5 min at 95°C and resolved on Tris-glycine SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by semi-dry blotting. Membranes were blocked in 4% milk in PBST and incubated overnight at 4°C with primary antibodies diluted in 4% milk/PBST. Next, membranes were washed 3x 10 min in PBST and incubated with secondary antibodies diluted in 4% milk/PBST

at room temperature for 1 h. Membranes were washed for 3x 10 min in PBST, and signals were detected using an Odyssey (LI-COR) imaging system. Signal intensities were measured with Fiji (Schindelin *et al*, 2012). Statistical analysis was done in GraphPad Prism 10.

The antibody against RPS27A/eS31 was described previously (Montellese *et al*., 2020). Other primary antibodies that were used in this study are: anti-β-actin (1:2000, Santa Cruz, sc-47778), anti-eRF1 (1:1000, Santa Cruz, sc-365686), anti-RNF25 (1:500, Santa Cruz, sc-398749), anti-GCN1 (1:1000, Bethyl, A301-843A), anti-GCN2 (1:1000, Abcam, ab134053), anti-phospho-GCN2 (Thr899) (1:500, Cell Signaling, 94668), anti-EDF1 (1:1000, Abcam, ab174651), anti-ZAKα (1:1000, Bethyl, A301-993A), anti-HA (1:1000, Enzo, ENZ-ABS120-0200). Antibodies against human DRG2 and RWDD1 were raised in rabbits against purified recombinant His_6-tagged proteins expressed in *E. coli*. Secondary antibodies used in this study were: goat anti-mouse IgG (H+L) Alexa Fluor™ 680 (1:10000, Invitrogen, A21058), goat anti-rabbit IgG (H+L) Alexa Fluor™ Plus 800 (1:10000, Invitrogen, A32735).

Sucrose density gradients

HeLa cells were grown on 10 cm plates and treated with 100 µg/ml cycloheximide (Sigma, C7698) for 10 min prior to harvesting. Cells were washed once with ice-cold 1x PBS containing 100 µg/ml cycloheximide and collected by scraping in 400 µl of lysis buffer (50 mM HEPES-KOH pH 7.5, 100 mM KAc, 10 mM $MgAc_2$, 0.5 % NP-40, 1 mM DTT, 100 µg/ml cycloheximide, 1 µg/ml pepstatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 40 U/ml RiboLock RNase inhibitor (Thermo Fisher, EO0381). Collected cells were incubated on ice for 5 min and the resulting lysates were cleared by centrifugation (16,000 x g, 10 min, 4°C). The A260 of lysates was measured with NanoDrop. Equal amounts of A260 units were loaded onto 10%-50% sucrose gradients produced in gradient buffer (50 mM HEPES-KOH pH 7.5, 100 mM KAc, 10 mM $MgAc_2$, 1 mM DTT, 100 µg/ml cycloheximide). Lysates were centrifuged in a SW41 rotor for 2 h at 39,000 rpm, and the A260 profiles of the gradients were monitored using a BioComp fractionator at the A260 during gradient fractionation. Areas under the monosome and polysome peaks were measured with Fiji (Schindelin *et al*., 2012), and statistical analysis was done in GraphPad Prism 10.

Visualization and modelling

Protein sequences were visualized in Jalview and aligned using the Clustal W (Procter *et al*, 2021; Waterhouse *et al*, 2009). Protein interactions were predicted using the AlphaFold 3 web server (Abramson *et al*, 2024).

Data availability

There are no datasets associated with this manuscript.

Acknowledgements

We thank Bianka Horváth for critical reading of the manuscript, members of the Kutay lab for helpful discussion, J. uit de Bos for advice on prime editing, I. Lazarevic and B. Widmann for generation of anti-DRG2 and anti-RWDD1 antibodies, and C. Montellese for initial work on RPS27A gene-editing. We are grateful to our colleagues C.M.T. Spahn and F. Wiechert (Universitätsmedizin Berlin) as well as O. Mühlemann and W. Teodorowicz (University of Bern) for helpful discussions and collaboration on related aspects.

Additional information

Funding

This work was supported by the Swiss National Science Foundation (SNSF) in the framework of the NCCR “RNA and disease” (51NF40-205601) and by the SNSF Advanced Grant TMAG-3_209245 to U.K..

Funding

Funder	Grant reference number	Author
Swiss National Science Foundation	TMAG-3_209245	Ulrike Kutay
Swiss National Science Foundation	51NF40-205601	Ulrike Kutay

Author ORCID iDs

Ulrike Kutay: https://orcid.org/0000-0002-8257-7465

Additional files

Supplemental Material.

References

1. **Abramson J,** Adler J, Dunger J, Evans R, Green T, Pritzel A, Ronneberger O, Willmore L, Ballard AJ, Bambrick J, *et al.* (2024) Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**:493-500 https://doi.org/10.1038/s41586-024-07487-w | PubMed
2. **Best K,** Ikeuchi K, Kater L, Best D, Musial J, Matsuo Y, Berninghausen O, Becker T, Inada T, Beckmann R (2023) Structural basis for clearing of ribosome collisions by the RQT complex. *Nat Commun* **14**:921 https://doi.org/10.1038/s41467-023-36230-8 | PubMed
3. **Brinkman EK,** Chen T, Amendola M, van Steensel B (2014) Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res* **42**:e168 https://doi.org/10.1093/nar/gku936 | PubMed
4. **Carelli JD,** Sethofer SG, Smith GA, Miller HR, Simard JL, Merrick WC, Jain RK, Ross NT, Taunton J (2015) Ternatin and improved synthetic variants kill cancer cells by targeting the elongation factor-1A ternary complex. *eLife* 4 https://doi.org/10.7554/elife.10222 | PubMed
5. **Castilho BA,** Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E (2014) Keeping the eIF2 alpha kinase Gcn2 in check. *Biochim Biophys Acta* **1843**:1948-1968 https://doi.org/10.1016/j.bbamcr.2014.04.006 | PubMed
6. **Chang WD,** Choe YJ (2026) Quality control and signaling pathways at stalled ribosomes. *Exp Mol Med* https://doi.org/10.1038/s12276-025-01623-w | PubMed
7. **Chen PJ,** Hussmann JA, Yan J, Knipping F, Ravisankar P, Chen PF, Chen C, Nelson JW, Newby GA, Sahin M, *et al.* (2021) Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. *Cell* **184**:5635-5652. https://doi.org/10.1016/j.cell.2021.09.018 | PubMed
8. **Choe YJ,** Park SH, Hassemer T, Korner R, Vincenz-Donnelly L, Hayer-Hartl M, Hartl FU (2016) Failure of RQC machinery causes protein aggregation and proteotoxic stress. *Nature* **531**:191-195 https://doi.org/10.1038/nature16973 | PubMed
9. **Chu J,** Hong NA, Masuda CA, Jenkins BV, Nelms KA, Goodnow CC, Glynne RJ, Wu H, Masliah E, Joazeiro CA, *et al.* (2009) A mouse forward genetics screen identifies LISTERIN as an E3 ubiquitin ligase involved in neurodegeneration. *Proc Natl Acad Sci U S A* **106**:2097-2103 https://doi.org/10.1073/pnas.0812819106 | PubMed
10. **Darnell AM,** Subramaniam AR, O'Shea EK (2018) Translational Control through Differential Ribosome Pausing during Amino Acid Limitation in Mammalian Cells. *Mol Cell* **71**:229-243. https://doi.org/10.1016/j.molcel.2018.06.041 | PubMed
11. **Defenouillere Q,** Zhang E, Namane A, Mouaikel J, Jacquier A, Fromont-Racine M (2016) Rqc1 and Ltn1 Prevent C-terminal Alanine-Threonine Tail (CAT-tail)-induced Protein Aggregation by Efficient Recruitment of Cdc48 on Stalled 60S Subunits. *J Biol Chem* **291**:12245-12253 https://doi.org/10.1074/jbc.m116.722264 | PubMed

12. **Doench JG,** Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, *et al.* (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* **34**:184-191 https://doi.org/10.1038/nbt.3437 | PubMed

13. **Doman JL,** Sousa AA, Randolph PB, Chen PJ, Liu DR (2022) Designing and executing prime editing experiments in mammalian cells. *Nat Protoc* **17**:2431-2468 https://doi.org/10.1038/s41596-022-00724-4 | PubMed

14. **Dougherty SE,** Maduka AO, Inada T, Silva GM (2020) Expanding Role of Ubiquitin in Translational Control. *Int J Mol Sci* 21 https://doi.org/10.3390/ijms21031151 | PubMed

15. **Fedry J,** Silva J, Vanevic M, Fronik S, Mechulam Y, Schmitt E, des Georges A, Faller WJ, Forster F (2024) Visualization of translation reorganization upon persistent ribosome collision stress in mammalian cells. *Mol Cell* **84**:1078-1089 e1074 https://doi.org/10.1016/j.molcel.2024.01.015 | PubMed

16. **Ford PW,** Narasimhan M, Bennett EJ (2024) Ubiquitin-dependent translation control mechanisms: Degradation and beyond. *Cell Rep* **43**:115050 https://doi.org/10.1016/j.celrep.2024.115050 | PubMed

17. **Garreau de Loubresse N,** Prokhorova I, Holtkamp W, Rodnina MV, Yusupova G, Yusupov M (2014) Structural basis for the inhibition of the eukaryotic ribosome. *Nature* **513**:517-522 https://doi.org/10.1038/nature13737

18. **Garshott DM,** Sundaramoorthy E, Leonard M, Bennett EJ (2020) Distinct regulatory ribosomal ubiquitylation events are reversible and hierarchically organized. *eLife* 9 https://doi.org/10.7554/elife.54023 | PubMed

19. **Geng J,** Li S, Li Y, Wu Z, Bhurtel S, Rimal S, Khan D, Ohja R, Brandman O, Lu B (2024) Stalled translation by mitochondrial stress upregulates a CNOT4-ZNF598 ribosomal quality control pathway important for tissue homeostasis. *Nat Commun* **15**:1637 https://doi.org/10.1038/s41467-024-45525-3 | PubMed

20. **Gurzeler LA,** Link M, Ibig Y, Schmidt I, Galuba O, Schoenbett J, Gasser-Didierlaurant C, Parker CN, Mao X, Bitsch F, *et al.* (2023) Drug-induced eRF1 degradation promotes readthrough and reveals a new branch of ribosome quality control. *Cell Rep* **42**:113056 https://doi.org/10.1016/j.celrep.2023.113056 | PubMed

21. **Hannus M,** Beitzinger M, Engelmann JC, Weickert MT, Spang R, Hannus S, Meister G (2014) siPools: highly complex but accurately defined siRNA pools eliminate off-target effects. *Nucleic Acids Res* **42**:8049-8061 https://doi.org/10.1093/nar/gku480 | PubMed

22. **Harding HP,** Ordonez A, Allen F, Parts L, Inglis AJ, Williams RL, Ron D (2019) The ribosomal P-stalk couples amino acid starvation to GCN2 activation in mammalian cells. *eLife* 8 https://doi.org/10.7554/elife.50149 | PubMed

23. **Hashimoto S,** Sugiyama T, Yamazaki R, Nobuta R, Inada T (2020) Identification of a novel trigger complex that facilitates ribosome-associated quality control in mammalian cells. *Sci Rep* **10**:3422 https://doi.org/10.1038/s41598-020-60241-w | PubMed

24. **Hinnebusch AG** (1994) The eIF-2 alpha kinases: regulators of protein synthesis in starvation and stress. *Semin Cell Biol* **5**:417-426 https://doi.org/10.1006/scel.1994.1049 | PubMed

25. **Hinnebusch AG** (2005) Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* **59**:407-450 https://doi.org/10.1146/annurev.micro.59.031805.133833 | PubMed

26. **Hsu JY,** Grunewald J, Szalay R, Shih J, Anzalone AV, Lam KC, Shen MW, Petri K, Liu DR, Joung JK, *et al.* (2021) PrimeDesign software for rapid and simplified design of prime editing guide RNAs. *Nat Commun* **12**:1034 https://doi.org/10.1038/s41467-021-21337-7 | PubMed

27. **Huso VL,** Niu S, Catipovic MA, Saba JA, Denk T, Park E, Cheng J, Berninghausen O, Becker T, Green R, *et al.* (2026) ZAK activation at the collided ribosome. *Nature* **649**:1051-1060 https://doi.org/10.1038/s41586-025-09772-8 | PubMed

28. **Ikeuchi K,** Tesina P, Matsuo Y, Sugiyama T, Cheng J, Saeki Y, Tanaka K, Becker T, Beckmann R, Inada T (2019) Collided ribosomes form a unique structural interface to induce Hel2-driven quality control pathways. *EMBO J* 38 https://doi.org/10.15252/embj.2018100276 | PubMed

29. **Ishikawa K,** Akiyama T, Ito K, Semba K, Inoue J (2009) Independent stabilizations of polysomal Drg1/Dfrp1 complex and non-polysomal Drg2/Dfrp2 complex in mammalian cells. *Biochem Biophys Res Commun* **390**:552-556 https://doi.org/10.1016/j.bbrc.2009.10.003 | PubMed

30. **Ishikawa K,** Ito K, Inoue J, Semba K (2013) Cell growth control by stable Rbg2/Gir2 complex formation under amino acid starvation. *Genes Cells* **18**:859-872 https://doi.org/10.1111/gtc.12082 | PubMed

31. **Ishimura R,** Nagy G, Dotu I, Chuang JH, Ackerman SL (2016) Activation of GCN2 kinase by ribosome stalling links translation elongation with translation initiation. *eLife* 5 https://doi.org/10.7554/elife.14295 | PubMed

32. **Itzhak DN,** Tyanova S, Cox J, Borner GH (2016) Global, quantitative and dynamic mapping of protein subcellular localization. *eLife* 5 https://doi.org/10.7554/elife.16950 | PubMed

33. **Izawa T,** Park SH, Zhao L, Hartl FU, Neupert W (2017) Cytosolic Protein Vms1 Links Ribosome Quality Control to Mitochondrial and Cellular Homeostasis. *Cell* **171**:890-903. https://doi.org/10.1016/j.cell.2017.10.002 | PubMed

34. **Joazeiro CAP** (2019) Mechanisms and functions of ribosome-associated protein quality control. *Nat Rev Mol Cell Biol* **20**:368-383 https://doi.org/10.1038/s41580-019-0118-2 | PubMed

35. **Juette MF,** Carelli JD, Rundlet EJ, Brown A, Shao S, Ferguson A, Wasserman MR, Holm M, Taunton J, Blanchard SC (2022) Didemnin B and ternatin-4 differentially inhibit conformational changes in eEF1A required for aminoacyl-tRNA accommodation into mammalian ribosomes. *eLife* 11 https://doi.org/10.7554/elife.81608 | PubMed

36. **Juszkiewicz S,** Chandrasekaran V, Lin Z, Kraatz S, Ramakrishnan V, Hegde RS (2018) ZNF598 Is a Quality Control Sensor of Collided Ribosomes. *Mol Cell* **72**:469-481. https://doi.org/10.1016/j.molcel.2018.08.037 | PubMed

37. **Juszkiewicz S,** Hegde RS (2017) Initiation of Quality Control during Poly(A) Translation Requires Site-Specific Ribosome Ubiquitination. *Mol Cell* **65**:743-750. https://doi.org/10.1016/j.molcel.2016.11.039 | PubMed

38. **Juszkiewicz S,** Slodkowicz G, Lin Z, Freire-Pritchett P, Peak-Chew SY, Hegde RS (2020a) Ribosome collisions trigger cis-acting feedback inhibition of translation initiation. *eLife* 9 https://doi.org/10.7554/elife.60038 | PubMed

39. **Juszkiewicz S,** Speldewinde SH, Wan L, Svejstrup JQ, Hegde RS (2020b) The ASC-1 Complex Disassembles Collided Ribosomes. *Mol Cell* **79**:603-614. https://doi.org/10.1016/j.molcel.2020.06.006 | PubMed

40. **Kim KQ,** Li JJ, Nanjaraj Urs AN, Pacheco ME, Lasehinde V, Denk T, Tesina P, Tomomatsu S, Matsuo Y, McDonald E, *et al.* (2024) Multiprotein bridging factor 1 is required for robust activation of the integrated stress response on collided ribosomes. *Mol Cell* **84**:4594-4611. https://doi.org/10.1016/j.molcel.2024.10.029 | PubMed

41. **Martin PB,** Kigoshi-Tansho Y, Sher RB, Ravenscroft G, Stauffer JE, Kumar R, Yonashiro R, Muller T, Griffith C, Allen W, *et al.* (2020) NEMF mutations that impair ribosome-associated quality control are associated with neuromuscular disease. *Nat Commun* **11**:4625 https://doi.org/10.1038/s41467-020-18327-6 | PubMed

42. **Marton MJ,** Crouch D, Hinnebusch AG (1993) GCN1, a translational activator of GCN4 in Saccharomyces cerevisiae, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2. *Mol Cell Biol* **13**:3541-3556 https://doi.org/10.1128/mcb.13.6.3541-3556.1993 | PubMed

43. **Masson GR** (2019) Towards a model of GCN2 activation. *Biochem Soc Trans* **47**:1481-1488 https://doi.org/10.1042/bst20190331 | PubMed

44. **Matsuo Y,** Ikeuchi K, Saeki Y, Iwasaki S, Schmidt C, Udagawa T, Sato F, Tsuchiya H, Becker T, Tanaka K, *et al.* (2017) Ubiquitination of stalled ribosome triggers ribosome-associated quality control. *Nat Commun* **8**:159 https://doi.org/10.1038/s41467-017-00188-1 | PubMed

45. **Matsuo Y,** Uchihashi T, Inada T (2023) Decoding of the ubiquitin code for clearance of colliding ribosomes by the RQT complex. *Nat Commun* **14**:79 https://doi.org/10.1038/s41467-022-35608-4 | PubMed

46. **Montellese C,** Ashiono C, Dorner K, Melnik A, Jonas S, Zemp I, Picotti P, Gillet LC, Kutay U (2020) USP16 counteracts mono-ubiquitination of RPS27a and promotes maturation of the 40S ribosomal subunit. *eLife* 9 https://doi.org/10.7554/elife.54435 | PubMed

47. **Muller MBD,** Kasturi P, Jayaraj GG, Hartl FU (2023) Mechanisms of readthrough mitigation reveal principles of GCN1-mediated translational quality control. *Cell* **186**:3227-3244. https://doi.org/10.1016/j.cell.2023.05.035 | PubMed

48. **Narita M,** Denk T, Matsuo Y, Sugiyama T, Kikuguchi C, Ito S, Sato N, Suzuki T, Hashimoto S, Machova I, *et al.* (2022) A distinct mammalian disome collision interface harbors K63-linked polyubiquitination of uS10 to trigger hRQT-mediated subunit dissociation. *Nat Commun* **13**:6411 https://doi.org/10.1038/s41467-022-34097-9 | PubMed

49. **Natchiar SK,** Myasnikov AG, Kratzat H, Hazemann I, Klaholz BP (2017) Visualization of chemical modifications in the human 80S ribosome structure. *Nature* **551**:472-477 https://doi.org/10.1038/nature24482 | PubMed

50. **Nelson JW,** Randolph PB, Shen SP, Everette KA, Chen PJ, Anzalone AV, An M, Newby GA, Chen JC, Hsu A, *et al.* (2022) Engineered pegRNAs improve prime editing efficiency. *Nat Biotechnol* **40**:402-410 https://doi.org/10.1038/s41587-021-01039-7 | PubMed

51. **Oltion K,** Carelli JD, Yang T, See SK, Wang HY, Kampmann M, Taunton J (2023) An E3 ligase network engages GCN1 to promote the degradation of translation factors on stalled ribosomes. *Cell* **186**:346-362. https://doi.org/10.1016/j.cell.2022.12.025 | PubMed

52. **Park J,** Park J, Lee J, Lim C (2021) The trinity of ribosome-associated quality control and stress signaling for proteostasis and neuronal physiology. *BMB Rep* **54**:439-450 https://doi.org/10.5483/bmbrep.2021.54.9.097 | PubMed

53. **Pereira CM,** Sattlegger E, Jiang HY, Longo BM, Jaqueta CB, Hinnebusch AG, Wek RC, Mello LE, Castilho BA (2005) IMPACT, a protein preferentially expressed in the mouse brain, binds GCN1 and inhibits GCN2 activation. *J Biol Chem* **280**:28316-28323 https://doi.org/10.1074/jbc.m408571200 | PubMed

54. **Pochopien AA,** Beckert B, Kasvandik S, Berninghausen O, Beckmann R, Tenson T, Wilson DN (2021) Structure of Gcn1 bound to stalled and colliding 80S ribosomes. *Proc Natl Acad Sci U S A* 118 https://doi.org/10.1073/pnas.2022756118 | PubMed

55. **Procter JB,** Carstairs GM, Soares B, Mourao K, Ofoegbu TC, Barton D, Lui L, Menard A, Sherstnev N, Roldan-Martinez D, *et al.* (2021) Alignment of Biological Sequences with Jalview. *Methods Mol Biol* **2231**:203-224 https://doi.org/10.1007/978-1-0716-1036-7_13 | PubMed

56. **Sattlegger E,** Hinnebusch AG (2000) Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells. *EMBO J* **19**:6622-6633 https://doi.org/10.1093/emboj/19.23.6622 | PubMed

57. **Schindelin J,** Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**:676-682 https://doi.org/10.1038/nmeth.2019 | PubMed

58. **Seidel AS,** Nemcekova L, Grønbæk-Thygesen M, Shi X, Ramalho S, Mordente KC, Bekker-Jensen S, Haahr P (2026) RNF25 restrains GCN2 hyperactivation to sustain protein synthesis and cell proliferation in response to RNA damage. *bioRxiv* https://doi.org/10.64898/2026.03.21.713335

59. **Sinha NK,** McKenney C, Yeow ZY, Li JJ, Nam KH, Yaron-Barir TM, Johnson JL, Huntsman EM, Cantley LC, Ordureau A, *et al.* (2024) The ribotoxic stress response drives UV-mediated cell death. *Cell* **187**:3652-3670. https://doi.org/10.1016/j.cell.2024.05.018 | PubMed

60. **Sinha NK,** Ordureau A, Best K, Saba JA, Zinshteyn B, Sundaramoorthy E, Fulzele A, Garshott DM, Denk T, Thoms M, *et al.* (2020) EDF1 coordinates cellular responses to ribosome collisions. *eLife* 9 https://doi.org/10.7554/elife.58828 | PubMed

61. **Stoneley M,** Harvey RF, Mulroney TE, Mordue R, Jukes-Jones R, Cain K, Lilley KS, Sawarkar R, Willis AE (2022) Unresolved stalled ribosome complexes restrict cell-cycle progression after genotoxic stress. *Mol Cell* **82**:1557-1572. https://doi.org/10.1016/j.molcel.2022.01.019 | PubMed

62. **Sundaramoorthy E,** Leonard M, Mak R, Liao J, Fulzele A, Bennett EJ (2017) ZNF598 and RACK1 Regulate Mammalian Ribosome-Associated Quality Control Function by Mediating Regulatory 40S Ribosomal Ubiquitylation. *Mol Cell* **65**:751-760. https://doi.org/10.1016/j.molcel.2016.12.026 | PubMed

63. **Thomas EN,** Kim KQ, McHugh EP, Marcinkiewicz T, Zaher HS (2020) Alkylative damage of mRNA leads to ribosome stalling and rescue by trans translation in bacteria. *eLife* 9 https://doi.org/10.7554/elife.61984 | PubMed

64. **Udagawa T,** Seki M, Okuyama T, Adachi S, Natsume T, Noguchi T, Matsuzawa A, Inada T (2021) Failure to Degrade CAT-Tailed Proteins Disrupts Neuronal Morphogenesis and Cell Survival. *Cell Rep* **34**:108599 https://doi.org/10.1016/j.celrep.2020.108599 | PubMed

65. **Vind AC,** Snieckute G, Blasius M, Tiedje C, Krogh N, Bekker-Jensen DB, Andersen KL, Nordgaard C, Tollenaere MAX, Lund AH, *et al.* (2020) ZAKalpha Recognizes Stalled Ribosomes through Partially Redundant Sensor Domains. *Mol Cell* **78**:700-713. https://doi.org/10.1016/j.molcel.2020.03.021 | PubMed

66. **Waller T,** Lee SJ, Sattlegger E (2012) Evidence that Yih1 resides in a complex with ribosomes. *Febs J* **279**:1761-1776 https://doi.org/10.1111/j.1742-4658.2012.08553.x | PubMed

67. **Waterhouse AM,** Procter JB, Martin DM, Clamp M, Barton GJ (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**:1189-1191 https://doi.org/10.1093/bioinformatics/btp033 | PubMed

68. **Welte T,** Tuck AC, Papasaikas P, Carl SH, Flemr M, Knuckles P, Rankova A, Buhler M, Grosshans H (2019) The RNA hairpin binder TRIM71 modulates alternative splicing by repressing MBNL1. *Genes Dev* **33**:1221-1235 https://doi.org/10.1101/gad.328492.119 | PubMed

69. **Wong W,** Bai XC, Brown A, Fernandez IS, Hanssen E, Condron M, Tan YH, Baum J, Scheres SH (2014) Cryo-EM structure of the Plasmodium falciparum 80S ribosome bound to the anti-protozoan drug emetine. *eLife* 3 https://doi.org/10.7554/elife.03080 | PubMed

70. **Worpenberg L,** Gobet C, Naef F (2025) Codon-specific ribosome stalling reshapes translational dynamics during branched-chain amino acid starvation. *Genome Biol* **26**:315 https://doi.org/10.1186/s13059-025-03800-6 | PubMed

71. **Wout PK,** Sattlegger E, Sullivan SM, Maddock JR (2009) Saccharomyces cerevisiae Rbg1 protein and its binding partner Gir2 interact on Polyribosomes with Gcn1. *Eukaryot Cell* **8**:1061-1071 https://doi.org/10.1128/ec.00356-08 | PubMed

72. **Wu CC,** Peterson A, Zinshteyn B, Regot S, Green R (2020) Ribosome Collisions Trigger General Stress Responses to Regulate Cell Fate. *Cell* **182**:404-416. https://doi.org/10.1016/j.cell.2020.06.006 | PubMed

73. **Wu CC,** Zinshteyn B, Wehner KA, Green R (2019a) High-Resolution Ribosome Profiling Defines Discrete Ribosome Elongation States and Translational Regulation during Cellular Stress. *Mol Cell* **73**:959-970. https://doi.org/10.1016/j.molcel.2018.12.009 | PubMed

74. **Wu Z,** Tantray I, Lim J, Chen S, Li Y, Davis Z, Sitron C, Dong J, Gispert S, Auburger G, *et al.* (2019b) MISTERMINATE Mechanistically Links Mitochondrial Dysfunction with Proteostasis Failure. *Mol Cell* **75**:835-848. https://doi.org/10.1016/j.molcel.2019.06.031 | PubMed

75. **Yan LL,** Zaher HS (2021) Ribosome quality control antagonizes the activation of the integrated stress response on colliding ribosomes. *Mol Cell* **81**:614-628. https://doi.org/10.1016/j.molcel.2020.11.033 | PubMed

76. **Yip MCJ,** Shao S (2021) Detecting and Rescuing Stalled Ribosomes. *Trends Biochem Sci* **46**:731-743 https://doi.org/10.1016/j.tibs.2021.03.008 | PubMed

77. **Zhao S,** Palma-Chaundler CS, Engel CM, Cordes J, Nixdorf D, Luo MY, Kaya S, Suryo Rahmanto A, Mackens-Kiani T, et al (2026) RNF25 confers mRNA damage tolerance by curbing activation of the integrated stress response. *Mol Cell* **86**:1275-1292. https://doi.org/10.1016/j.molcel.2026.02.024 | PubMed

78. **Zhou C,** Zhang M, Murray J, Paulo J, Gygi S, Shao S, Whitman M, Keller T (2025) GCN1 couples GCN2 to ribosomal state to initiate amino acid response pathway signaling. *Science* **390**:eads8728 https://doi.org/10.1126/science.ads8728 | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

In this manuscript, the authors investigate ubiquitylation of RPS27A/eS31 by the E3 ligase RNF25 in response to translational stress. Previous studies have identified RPS27A/eS31 ubiquitylation at Lys113 under conditions where translation factors are trapped in the ribosomal A-site. Here, the authors extend this work by testing whether additional translational stress conditions, including amino acid deprivation, induce RPS27A/eS31 ubiquitylation. They further show that GCN1 is required and explore a possible competition between RNF25 and GCN2 for GCN1.

Strengths:

This study expands on the range of stress conditions leading to RPS27A/eS31 ubiquitylation, reporting that it occurs in a variety of conditions associated with ribosome stalling, including amino acid deprivation. These observations are useful because they suggest that the RNF25 pathway may not require translation factors trapped in the ribosomal A-site, but may instead respond more broadly to translational perturbations associated with ribosome collisions.

Weaknesses:

The evidence supporting several of the major claims is incomplete, and additional controls and orthogonal approaches would greatly strengthen the evidence presented. In particular:

(1) It is unclear whether the different conditions used to induce translational stress lead to ribosome stalling or collisions. The model presented by the authors seems to rely on ribosomal collisions, but this is not shown. In addition, further investigating amino acid deprivation beyond the removal of Arg or Lys would strengthen the paper.

(2) Ubiquitylation of RPS27A/eS31 by RNF25 is used throughout the paper as a readout of RNF25 activity and is assumed to be on Lys113 based on previous work, but is not formally shown here.

(3) Rescue experiments of the different mutants used in this study with wild-type and different domain deletions (i.e., ΔRWD for RNF25, ΔRWD-binding for GCN1) would help confirm specificity and strengthen the mechanistic claims.

(4) The conclusion that RPS27A/eS31 ubiquitylation supports translation (Figure 4) is based entirely on polysome/monosome ratios, which are difficult to interpret without additional assays of translation output, elongation, or collision.

(5) The idea that RNF25 competes with GCN2 for GCN1 binding is interesting, and related models have recently been proposed in RNA damage. The effect of GCN2 KO on RNF25-dependent ubiquitylation appears modest, and the data would be strengthened by rescue experiments with wild-type GCN2 and GCN2 mutants defective in GCN1 binding. The authors propose: "that the RNF25 pathway acts as a first line of defence to resolve ribosome collisions, outcompeted by GCN2 binding to GCN1 under acute stress." This model would suggest a

further increase in RPS27A/eS31 ubiquitylation upon Arg/Lys deprivation in GCN2 KO cells, since this is the condition in which GCN2 is expected to be activated and engaged with GCN1 (i.e., when it would be competing with RNF25), but no further increase in RPS27A ubiquitylation is observed. It is therefore not clear that these data support the proposed model. Contributing to this may be the fact that many of these assays are performed in a USP16 KO background, which may make it difficult to assess changes in RPS27A/eS31 ubiquitylation.

(6) Given that several RWD domain proteins can interact with GCN1, and that DRG2 KO appears to affect RPS27A/eS31 ubiquitylation (Figure S5), the data do not support the GCN2-specific title. The results are more consistent with a broader, incompletely characterized network of GCN1-associated RWD domain-containing proteins that seems to affect RNF25-dependent ubiquitylation rather than with a demonstrated RNF25-GCN2 competition mechanism. Further characterization of GCN2-dependent ISR activation (p-eIF2a and ATF4 WB) in the absence of RNF25 in Arg/Lys starvation will help shed light on the RNF25-GCN2 competition. The authors use K113R, but this is not shown to prevent RNF25 engagement with GCN1, so a RNF25 KO should be used.

Overall, the study contains useful observations, but the mechanistic claims are not yet fully supported.

https://doi.org/10.7554/eLife.112045.1.sa3

Reviewer #2 (Public review):

Summary:

The authors show that deprivation of Arginine and Lysine induces a ~50% increase in the ratio of ubi-RPS27A to RPS27A, and this induction requires E3 ubiquitin ligase RNF25. The authors show ZAKalpha and EDF1 are not required for steady state or ribosome stalling-induced ubi-RPS27A, while GCN1 is required. The ratio of polysomes to monosomes is increased in RNF25 knockdown cells or when translation is activated by ISRIB in a RPS27A K113R mutant cell line. GCN2 KO cells indicate elevated levels of ubi-RPS27A, and overexpression of the GCN2 RWD domain reduces levels of ubi-RPS27A.

Strengths:

(1) The authors identified a novel pathway to sense amino acid deprivation, indicated by ubi-RPS27A, previously implicated in ribosome stalling.

(2) The authors find antagonism between two proteins known to act downstream of GCN1, giving insight into how signaling occurs from an upstream sensor of ribosome stalling to multiple downstream pathways.

Weaknesses:

(1) The authors suggest that, based on increased Polysome/Monosome ratios, there is more disome stalling in RNF25 KD cells and RPS27A K113R cells treated with ISRIB, but this readout is very indirect and could be driven by other changes in the cell other than ribosome stalling.

(2) While the authors propose that GCN2 and RNF25 compete for binding to GCN1, no evidence was shown that RNF25 binds to GCN1 in cells, nor that the interaction increases when GCN2 is absent.

(3) The use of USP16 to enhance the detection of ubi-RPS27A in many experiments brings the question of whether USP16 KO may alter the protein levels of any known regulators of ribosome collisions? (i.e. ZNF598, GCN1, EDF1, ZAKalpha, etc.) If USP16 KO causes changes in

other important regulators of collisions, the authors could be identifying genetic interactions with USP16 in their experiments throughout the paper.

(4) In Figure 5E, the expression level of the GCN2 3K RWD domain looks to be lower than the WT RWD domain; perhaps this could be what is driving the smaller decrease of ubi-RPS27A seen with GCN2 3K vs WT.

https://doi.org/10.7554/eLife.112045.1.sa2

Reviewer #3 (Public review):

Summary:

This study examines the role of RNF25 in translational quality control. Previous work indicated that RNF25 is activated by ribosomes stalled with defective elongation or termination factors bound in the A-site. Here, the authors provide evidence that RNF25 is activated by other treatments that evoke ribosome stalling, including amino acid starvation, where the A-site may be empty, leading to ubiquitination of RPS27A in a manner requiring the ISR collision sensor Gcn1, but not EDF1 and ZAKα, involved in the RQC and RSR surveillance pathways. They present some evidence from polysome profiling that RNF25 and its ubiquitination of RPS7A help resolve ribosome collisions and support translation elongation in basal conditions. They further show that KO of Gcn2 increases RPS27A ubiquitination in basal conditions, but not in amino acid-starved cells, and that RPS27A ubiquitination was reduced on overexpressing the WT RWD domain of Gcn2 but not a variant harboring substitutions of residues predicted to bind Gcn1. Based on these findings, they propose a model that, in response to ribosome stalling induced by various stresses, Gcn1 recruits RNF25 via the latter's RWD domain to ubiquitinate RPS27A and thereby resolve ribosome stalling and promote continued elongation. If collisions increase even further, GCN1 recruits GCN2 instead of RNF25 to elicit the ISR.

Strengths:

The data is convincing that a variety of triggers leading to diverse stalled ribosomal states, including amino acid limitation, can activate RNF25, suggesting that activation of this pathway does not require the presence of trapped protein factors in the ribosomal A-site but is a more general response to ribosome collisions. It is also convincing that Gcn1 is required for RNF25 activation under all of these conditions, which is consistent with previous findings that Gcn1 is required for RNF25 function in the presence of trapped elongation or termination factors. The finding that EDF1 and ZAK are not needed for RNF25 activation in amino acid starvation conditions is of interest for EDF1, given the recent claim that it is required for full ISR activation.

Weaknesses:

The evidence presented from polysome profiling that RNF25 helps resolve naturally occurring ribosome collisions in basal conditions is not compelling, as eliminating RNF25 could be increasing the rate of initiation rather than increasing stalled ribosomes as the means of increasing the P/M ratio. The Rps27A-K113R mutation could have the same effect of increasing initiation, which could have been obscured by inhibiting the ISR with ISRIB.

The evidence that RNF25 competes with Gcn2 for Gcn1 binding is also not compelling. While it's convincing that Rps27A-Ubi is elevated in basal conditions on eliminating Gcn2, loss of GCN2 would be expected to increase ribosome loading on mRNAs, potentially elevating the frequency of collisions and thereby stimulating RNF25 activity indirectly.

It's also quite puzzling and left unexplained why they observed no further increase in Rps27A-Ubi on -Arg/-Lys starvation in the cells lacking Gcn2. Why wouldn't -Arg/-Lys

starvation lead to further stalling and RNF25 activation in the absence of Gcn2? (Since Gcn2 KO increases Rps27A-Ubi in the presence +Arg/+Lys conditions, it can't be that Gcn2 is required for RNF25 function.) The same puzzling and unresolved observation was made in the cells lacking DRG2. One possible explanation for this conundrum is that low-level RNF25 abundance limits further activation.

The quantitative effects of overexpressing the Gcn2 RWD domain on Rps27A-Ubi, constituting their other evidence presented to support the competition model, are quite small in magnitude.

https://doi.org/10.7554/eLife.112045.1.sa1

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *In this manuscript, the authors investigate ubiquitylation of RPS27A/eS31 by the E3 ligase RNF25 in response to translational stress. Previous studies have identified RPS27A/eS31 ubiquitylation at Lys113 under conditions where translation factors are trapped in the ribosomal A-site. Here, the authors extend this work by testing whether additional translational stress conditions, including amino acid deprivation, induce RPS27A/eS31 ubiquitylation. They further show that GCN1 is required and explore a possible competition between RNF25 and GCN2 for GCN1.*
>
> *Strengths:*
>
> *This study expands on the range of stress conditions leading to RPS27A/eS31 ubiquitylation, reporting that it occurs in a variety of conditions associated with ribosome stalling, including amino acid deprivation. These observations are useful because they suggest that the RNF25 pathway may not require translation factors trapped in the ribosomal A-site, but may instead respond more broadly to translational perturbations associated with ribosome collisions.*

We wish to point out that our study in fact suggests that the RNF25 pathway is activated by translation factors in the A-site, in agreement with what has been previously proposed, and in addition by stalling conditions that are assumed to not trap translation factors in the A-site. We do not exclude that these conditions might be sampled by A-site binding quality control factors before recognition by RNF25.

> *Weaknesses:*
>
> *The evidence supporting several of the major claims is incomplete, and additional controls and orthogonal approaches would greatly strengthen the evidence presented.*

We appreciate adding more controls to further substantiate our novel findings. In the course of the revisions we will focus our work on those experiments that do not merely reproduce established facts in the field.

> *In particular:*
>
> *(1) It is unclear whether the different conditions used to induce translational stress lead to ribosome stalling or collisions. The model presented by the authors seems to rely on ribosomal collisions, but this is not shown. In addition, further investigating amino acid deprivation beyond the removal of Arg or Lys would strengthen the paper.*

We thank the reviewer for the comment. It is correct that we don't formally show collisions.

However, the conditions we use have been previously established in the field to induce ribosome stalls and/or collisions, which we may not have pointed out clearly enough. In the revised version, we will include all relevant citations, i.e. for ternatin (Oltion et al., 2023): collisions, anisomycin (Juszkiewicz et al., 2018, Sinha et al., 2020): collisions, emetine (Sinha et al., 2020): collisions, didemnin B (Juszkiewicz et al., 2018, Stoneley et al., 2022): accumulation of ubi-eS10 and changes in polysome profiles indicative of collisions, MMS (Stoneley et al., 2022): changes in polysome profiles indicative of stalls or collisions, starvation -Arg/-Lys (Darnell et al., 2018, Stoneley et al., 2022): accumulation of collided ribosomes only upon GCN2 inhibition, indicative of collisions.

Secondly, we do not claim to induce collisions when describing the inhibition data (Figure 1 and Figure S1) and were careful to say that we use 'conditions that cause ribosome stalling'.

Thirdly, we conclude on collisions when interpreting the data on amino acid starvation (and in our model (Figure 6)), based on our data demonstrating that RNF25 activity in RPS27A/eS31 ubiquitylation is dependent on GCN1 (Figure 3), an established sensor of collided disomes (Pochopien et al., 2021). This conclusion is thus based on the current knowledge in the field.

We will carefully screen the text for potential points of overinterpretation or confusion between stalling and collisions.

To address the request of further investigating amino acid deprivation beyond the removal of Arg or Lys, we will include an additional experiment in which we will deplete another amino acid.

> *(2) Ubiquitylation of RPS27A/eS31 by RNF25 is used throughout the paper as a readout of RNF25 activity and is assumed to be on Lys113 based on previous work, but is not formally shown here.*

It is established that Lys113 is the main target of RNF25, not only by our work (Montellese et al., 2020), but also by recent work of other groups to which we had referred in our manuscript (Gurzeler et al., 2023, Oltion et al., 2023, Zhao et al., 2026).

To experimentally address this point, we will add an experiment testing ubiquitylation of RPS27A/eS31 in cells carrying the K113R mutation.

> *(3) Rescue experiments of the different mutants used in this study with wild-type and different domain deletions (i.e., ΔRWD for RNF25, ΔRWD-binding for GCN1) would help confirm specificity and strengthen the mechanistic claims.*

Minimally, we will include rescue experiments for RNF25 (using WT, DRWD and enzymatically dead mutant) and, if possible, also for GCN1, which might be more challenging due to its large size and anticipated problems with cloning, cell line generation and protein expression.

> *(4) The conclusion that RPS27A/eS31 ubiquitylation supports translation (Figure 4) is based entirely on polysome/monosome ratios, which are difficult to interpret without additional assays of translation output, elongation, or collision.*

It is correct that we base our conclusion on polysome profiles and agree that these are an indirect measure of translation output. However, this assay is well established in the field to show dysregulation of polysome/monosome ratio upon ribosome stalling (Garzia et al., 2017), (Wu et al., 2020), (Chatterjee et al., 2024), (Gurzeler et al., 2023).

Elongation defects would be expected to lead to stalls and/or collisions (which we conclude on). However, we cannot exclude that there is more initiation when RPS27A/eS31 carries the K113R mutation, although this is hard to rationalize mechanistically and experimentally challenging to exclude. Therefore, to address the point, we will add a sentence that we cannot exclude indirect effects on initiation but consider these unlikely.

> *(5) The idea that RNF25 competes with GCN2 for GCN1 binding is interesting, and related models have recently been proposed in RNA damage. The effect of GCN2 KO on RNF25dependent ubiquitylation appears modest, and the data would be strengthened by rescue experiments with wild-type GCN2 and GCN2 mutants defective in GCN1 binding. The authors propose: "that the RNF25 pathway acts as a first line of defence to resolve ribosome collisions, outcompeted by GCN2 binding to GCN1 under acute stress." This model would suggest a further increase in RPS27A/eS31 ubiquitylation upon Arg/Lys deprivation in GCN2 KO cells, since this is the condition in which GCN2 is expected to be activated and engaged with GCN1 (i.e., when it would be competing with RNF25), but no further increase in RPS27A ubiquitylation is observed. It is therefore not clear that these data support the proposed model. Contributing to this may be the fact that many of these assays are performed in a USP16 KO background, which may make it difficult to assess changes in RPS27A/eS31 ubiquitylation.*

We thank the reviewer for the comment. We measure on average a 50% increase in the level of ubiquitinated RPS27A/eS31 in *GCN2* KO cells. Considering the large number of ribosomes in a cell (~10^7 per HeLa cell), this 50% increase (from 12.5 to 25% ubiquitinated RPS27A/eS31) amounts to an estimated number of 1,25 x 10^6 of RPS27A/eS31 molecules that get additionally modified, which is clearly a substantial difference, especially compared to the naturally very low levels of RNF25 (in the range of 23'000 molecules (Itzhak et al., 2016)).

We respectfully disagree that performing experiments in *USP16* KO background makes it difficult to assess RPS27A/eS31 ubiquitination. On the contrary. The natural levels of RPS27A/eS31 ubiquitination in WT cells are very low, making quantification sensitive to background fluctuations (see Figure S1). Therefore, in our experience, the usage of USP16 KO makes the quantitative analysis of RPS27A/eS31 ubiquitination robust, allowing us to analyse both increase and decrease in the levels of ubiquitination. We agree that with increasing collisions, the level of ubiquitinated RPS27A/eS31 reaches a plateau in USP16 KO, which may limit the observable increase. Therefore, the substantial 50% increase might indeed underestimate the effect as compared to WT cells. Still, the measurable increase is substantial and robust.

To experimentally address the point of the reviewer, we will try generating *GCN2* KO cells in a WT background, i.e. in absence of *USP16* KO, to strengthen our model.

> *(6) Given that several RWD domain proteins can interact with GCN1, and that DRG2 KO appears to affect RPS27A/eS31 ubiquitylation (Figure S5), the data do not support the GCN2specific title. The results are more consistent with a broader, incompletely characterized network of GCN1-associated RWD domain-containing proteins that seems to affect RNF25-dependent ubiquitylation rather than with a demonstrated RNF25-GCN2 competition mechanism. Further characterization of GCN2-dependent ISR activation (p-eIF2a and ATF4 WB) in the absence of RNF25 in Arg/Lys starvation will help shed light on the RNF25-GCN2 competition. The authors use K113R, but this is not shown to prevent RNF25 engagement with GCN1, so a RNF25 KO should be used.*

While we fully agree that our data point at a broader network of competition on GCN1, we wished to avoid an overstatement on other pathways than GCN2, since our experimental evidence on DRG2 is limited at the moment. As it stands, changing the title of the manuscript to a more general message, would indeed fuel the view that our claims are incomplete. But we are glad to reconsider this suggestion if further supporting evidence can be obtained in the course of the revision work.

The reviewer suggests experiments on competition of RNF25 with GCN2. In contrast to the expectation of the reviewer, we do not expect KO of *RNF25* to manifest in defects in ISR activation due to the low expression levels of RNF25. In the revised manuscript, we will make clearer that our model refers to competition in the other direction, i.e., of GCN2 with RNF25, which our data supports. The reverse competition of RNF25 with GCN2 is expected to be inefficient to enable a robust activation of the ISR by GCN1 when needed. In addition, other pathways (such as DRG2) might also contribute to the resolution of collisions in the absence of RNF25, affecting the level of ISR activation.

We feel that further working out these competitive relationships will be interesting to perform in future work. Currently, it is also not clear whether all involved RWD-containing factors bind GCN1 with the same affinity, which is important to consider for the effectiveness of a mutual competition model as suggested by the reviewer.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The authors show that deprivation of Arginine and Lysine induces a ~50% increase in the ratio of ubi-RPS27A to RPS27A, and this induction requires E3 ubiquitin ligase RNF25. The authors show ZAKalpha and EDF1 are not required for steady state or ribosome stalling-induced ubiRPS27A, while GCN1 is required. The ratio of polysomes to monosomes is increased in RNF25 knockdown cells or when translation is activated by ISRIB in a RPS27A K113R mutant cell line. GCN2 KO cells indicate elevated levels of ubi-RPS27A, and overexpression of the GCN2 RWD domain reduces levels of ubi-RPS27A.*
>
> *Strengths:*
>
> *(1) The authors identified a novel pathway to sense amino acid deprivation, indicated by ubiRPS27A, previously implicated in ribosome stalling.*
>
> *(2) The authors find antagonism between two proteins known to act downstream of GCN1, giving insight into how signaling occurs from an upstream sensor of ribosome stalling to multiple downstream pathways.*
>
> *Weaknesses:*
>
> *(1) The authors suggest that, based on increased Polysome/Monosome ratios, there is more disome stalling in RNF25 KD cells and RPS27A K113R cells treated with ISRIB, but this readout is very indirect and could be driven by other changes in the cell other than ribosome stalling.*

We thank the reviewer for this important comment. We intentionally used ISRIB in Figure 4F, G to avoid possible effects on initiation, and the results are consistent with our model. While we agree that ISRIB itself might have indirect consequences, these should be the same for the control (WT cells) and the assay condition (K113R cells). We also show the data without ISRIB, which show a similar trend but are less robust (Figure 4D, E). It is very hard to exclude other possible effects which would selectively affect K113R cells in presence of ISRIB.

> *(2) While the authors propose that GCN2 and RNF25 compete for binding to GCN1, no evidence was shown that RNF25 binds to GCN1 in cells, nor that the interaction increases when GCN2 is absent.*

The idea of RNF25 binding to GCN1 is based on a previously published work (Oltion et al., 2023, Seidel et al., 2026, Zhao et al., 2026). We will design additional experiments to potentially confirm the interaction between RNF25 and GCN1.

> *(3) The use of USP16 to enhance the detection of ubi-RPS27A in many experiments brings the question of whether USP16 KO may alter the protein levels of any known regulators of ribosome collisions? (i.e. ZNF598, GCN1, EDF1, ZAKalpha, etc.) If USP16 KO causes changes in other important regulators of collisions, the authors could be identifying genetic interactions with USP16 in their experiments throughout the paper.*

Indeed, we can't exclude the effect of USP16 KO on the expression levels of other collision sensors. We will experimentally confirm the levels of other ribosome collision sensors in USP16 KO cells.

> *(4) In Figure 5E, the expression level of the GCN2 3K RWD domain looks to be lower than the WT RWD domain; perhaps this could be what is driving the smaller decrease of ubi-RPS27A seen with GCN2 3K vs WT.*

We thank the reviewer for pointing at this issue, which we will experimentally address in the revised version.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *This study examines the role of RNF25 in translational quality control. Previous work indicated that RNF25 is activated by ribosomes stalled with defective elongation or termination factors bound in the A-site. Here, the authors provide evidence that RNF25 is activated by other treatments that evoke ribosome stalling, including amino acid starvation, where the A-site may be empty, leading to ubiquitination of RPS27A in a manner requiring the ISR collision sensor Gcn1, but not EDF1 and ZAKa, involved in the RQC and RSR surveillance pathways. They present some evidence from polysome profiling that RNF25 and its ubiquitination of RPS7A help resolve ribosome collisions and support translation elongation in basal conditions. They further show that KO of Gcn2 increases RPS27A ubiquitination in basal conditions, but not in amino acid-starved cells, and that RPS27A ubiquitination was reduced on overexpressing the WT RWD domain of Gcn2 but not a variant harboring substitutions of residues predicted to bind Gcn1. Based on these findings, they propose a model that, in response to ribosome stalling induced by various stresses, Gcn1 recruits RNF25 via the latter's RWD domain to ubiquitinate RPS27A and thereby resolve ribosome stalling and promote continued elongation. If collisions increase even further, GCN1 recruits GCN2 instead of RNF25 to elicit the ISR.*
>
> *Strengths:*
>
> *The data is convincing that a variety of triggers leading to diverse stalled ribosomal states, including amino acid limitation, can activate RNF25, suggesting that activation of this pathway does not require the presence of trapped protein factors in the ribosomal A-site but is a more general response to ribosome collisions. It is also convincing that Gcn1 is required for RNF25 activation under all of these conditions, which is consistent with previous findings that Gcn1 is required for RNF25 function in the presence of trapped elongation or termination factors. The finding that EDF1 and ZAK are not needed for*

> *RNF25 activation in amino acid starvation conditions is of interest for EDF1, given the recent claim that it is required for full ISR activation.*
>
> *Weaknesses:*
>
> *(1) The evidence presented from polysome profiling that RNF25 helps resolve naturally occurring ribosome collisions in basal conditions is not compelling, as eliminating RNF25 could be increasing the rate of initiation rather than increasing stalled ribosomes as the means of increasing the P/M ratio. The Rps27A-K113R mutation could have the same effect of increasing initiation, which could have been obscured by inhibiting the ISR with ISRIB.*

Our results indicate that P/M ratio increases upon ISRIB treatment of K113R cells compared to WT cells, aligning with the idea that ISRIB enhances initiation, causing increased loading of ribosomes on mRNA and consequent increased frequency of collisions. As outlined above, we agree that this experiment is indirect and results might be affected by secondary effects. However, we cannot rationalize how inhibition of the ISR by ISRIB would specifically obscure the effect for the K113R mutation but not the WT.

> *(2) The evidence that RNF25 competes with Gcn2 for Gcn1 binding is also not compelling. While it's convincing that Rps27A-Ubi is elevated in basal conditions on eliminating Gcn2, loss of GCN2 would be expected to increase ribosome loading on mRNAs, potentially elevating the frequency of collisions and thereby stimulating RNF25 activity indirectly.*

We have not made sufficiently clear that we did not intend to claim that RNF25 efficiently competes with GCN2 (see also response to reviewer 1), which we do not expect due to the low levels of RNF25. Our manuscript is focussed on competition in the reverse direction, i.e. of GCN2 with RNF25.

We agree that loss of GCN2 may increase ribosome loading on mRNA similar to ISRIB treatment, which could lead to more collisions by enhanced translation and hence increased Rps27A-Ubi. At the same time, however, this does not exclude that loss of GCN2 contributes more directly at the level of RNF25 recruitment. Therefore, the experiment also supports the competition model, and both effects together may contribute to the observed increase in ubiquitylated RPS27A/eS31. Without other evidence, the experiment would remain inconclusive.

Therefore, to directly test the competition model, we had overexpressed the GCN1-binding RWD domain of GCN2, which leads to decreased levels of ubiquitinated RPS27A/eS31, lending direct support to the competition model of GCN2 with RNF25, which is consistent with similar models recently proposed by two other manuscripts (Seidel et al., 2026, Zhao et al., 2026).

> *(3) It's also quite puzzling and left unexplained why they observed no further increase in Rps27AUbi on -Arg/-Lys starvation in the cells lacking Gcn2. Why wouldn't -Arg/-Lys starvation lead to further stalling and RNF25 activation in the absence of Gcn2? (Since Gcn2 KO increases Rps27A-Ubi in the presence +Arg/+Lys conditions, it can't be that Gcn2 is required for RNF25 function.) The same puzzling and unresolved observation was made in the cells lacking DRG2. One possible explanation for this conundrum is that low-level RNF25 abundance limits further activation.*

Over all of our experiments, we have observed that RPS27A-Ubi reaches a plateau of about 30% to 35% of total RPS27A in the *USP16* KO background (GCN2 deletion or amino acid starvation). This plateau indeed limits seeing further increases. We do not know the underlying reason but note that under these conditions about one third of 40S subunits carry ubiquitin on RPS27A/eS31. As the reviewer suggests, RNF25 is expressed at low levels (in the range of 23'000 molecules, (Itzhak et al., 2016); see point 5 of reviewer 1), likely rendering it the limiting factor for further ubiquitination events.

To circumvent the plateau issue, we will attempt to generate *GCN2* KO cell lines in the WT background for the starvation experiments (see also response to reviewer 1, point 5).

> *(4) The quantitative effects of overexpressing the Gcn2 RWD domain on Rps27A-Ubi, constituting their other evidence presented to support the competition model, are quite small in magnitude.*

We respectfully disagree with the reviewers' comment concerning the magnitude of the effect. There is a ~27% decrease in ubiquitination, which is substantial considering the number of 40S ribosomal subunits and possible consequences of such change. It should also be noted that this is a transient transfection experiment not hitting all cells of the population. We will repeat the experiment, optimizing the expression of the negative control construct.

Cited literature:

Chatterjee S, Naeli P, Onar O, Simms N, Garzia A, Hackett A, Coyle K, Harris Snell P, McGirr T, Sawant TN et al. (2024) Ribosome Quality Control mitigates the cytotoxicity of ribosome collisions induced by 5-Fluorouracil. Nucleic Acids Res 52: 12534-12548

Darnell AM, Subramaniam AR, O'Shea EK (2018) Translational Control through Differential Ribosome Pausing during Amino Acid Limitation in Mammalian Cells. Mol Cell 71: 229-243 e11

Garzia A, Jafarnejad SM, Meyer C, Chapat C, Gogakos T, Morozov P, Amiri M, Shapiro M, Molina H, Tuschl T et al. (2017) The E3 ubiquitin ligase and RNA-binding protein ZNF598 orchestrates ribosome quality control of premature polyadenylated mRNAs. Nat Commun 8: 16056

Gurzeler LA, Link M, Ibig Y, Schmidt I, Galuba O, Schoenbett J, Gasser-Didierlaurant C, Parker CN, Mao X, Bitsch F et al. (2023) Drug-induced eRF1 degradation promotes readthrough and reveals a new branch of ribosome quality control. Cell Rep 42: 113056

Itzhak DN, Tyanova S, Cox J, Borner GH (2016) Global, quantitative and dynamic mapping of protein subcellular localization. Elife 5

Juszkiewicz S, Chandrasekaran V, Lin Z, Kraatz S, Ramakrishnan V, Hegde RS (2018) ZNF598 Is a Quality Control Sensor of Collided Ribosomes. Mol Cell 72: 469-481 e7

Montellese C, van den Heuvel J, Ashiono C, Dorner K, Melnik A, Jonas S, Zemp I, Picotti P, Gillet LC, Kutay U (2020) USP16 counteracts mono-ubiquitination of RPS27a and promotes maturation of the 40S ribosomal subunit. Elife 9

Oltion K, Carelli JD, Yang T, See SK, Wang HY, Kampmann M, Taunton J (2023) An E3 ligase network engages GCN1 to promote the degradation of translation factors on stalled ribosomes. Cell 186: 346-362 e17

Pochopien AA, Beckert B, Kasvandik S, Berninghausen O, Beckmann R, Tenson T, Wilson DN (2021) Structure of Gcn1 bound to stalled and colliding 80S ribosomes. Proc Natl Acad Sci U S A 118

Seidel AS, Nemcekova L, Grønbæk-Thygesen M, Shi X, Ramalho S, Mordente KC, Bekker-Jensen S, Haahr P (2026) RNF25 restrains GCN2 hyperactivation to sustain protein synthesis and cell proliferation in response to RNA damage. bioRxiv

Sinha NK, Ordureau A, Best K, Saba JA, Zinshteyn B, Sundaramoorthy E, Fulzele A, Garshott DM, Denk T, Thoms M et al. (2020) EDF1 coordinates cellular responses to ribosome collisions. Elife 9

Stoneley M, Harvey RF, Mulroney TE, Mordue R, Jukes-Jones R, Cain K, Lilley KS, Sawarkar R, Willis AE (2022) Unresolved stalled ribosome complexes restrict cell-cycle progression after genotoxic stress. Mol Cell 82: 1557-1572 e7

Wu CC, Peterson A, Zinshteyn B, Regot S, Green R (2020) Ribosome Collisions Trigger General Stress Responses to Regulate Cell Fate. Cell 182: 404-416 e14

Zhao S, Palma-Chaundler CS, Engel CM, Cordes J, Nixdorf D, Luo MY, Kaya S, Suryo Rahmanto A, van den Heuvel D, Mackens-Kiani T et al. (2026) RNF25 confers mRNA damage tolerance by curbing activation of the integrated stress response. Mol Cell 86: 1275-1292 e12

https://doi.org/10.7554/eLife.112045.1.sa0