nature portfolio

Peer Review File

Conserved GTPase OLA1 promotes efficient translation on D/E-rich mRNA

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.
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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript “Structure and regulation mechanism of Ola1 on the translation pausing” reports the structure of the highly conserved GTPase Ola1, which is a member of the TRAFAC (translation factors) class of GTPases. The molecular mechanism and cellular function of Ola1 and its bacterial homolog YchF have been a mystery since its discovery several decades ago. Many studies have reported critical roles in various cellular stress responses, and it has been implicated in the regulation of tumor progression in numerous types of cancers in Eukaryotes. Many studies have indicated a ribosome associated role and although structural information for the protein is available form a wide range of organisms, a complex bound to the ribosome is still missing. Recent biochemical studies using UV inducible crosslinkers indeed support binding of the YchF/Ola1 to the ribosome, crosslinks primary mapping to the E-site on the 30S subunit.

Here the authors report the structure of E. coli YchF bound to the 50S ribosomal subunit as detrei9mined by cryo-electron microscopy. This structure places the YchF into the A-site of the 50S subunit close to the translation-factor (EF-Tu / EF-G) binding site. To obtain this complex the authors used several different conditions including different non-hydrolysable analogs of ATP and GTP. Previous work has shown that Ola1/YchF although member of the GTPase family has a drastically altered Nucleotide binding pocket resulting in the different specificities for GTP and ATP depending on the organism. On this background the authors observed only weak binding to the ribosome (< 30% to the 70S ribosome) when in vitro reconstructing the complexes. The authors then proceed to directly purify the ribosome complex using the His-Tagged version of a previously reported hydrolysis inactive variant of the YchF (YchF H114A) form the delta YchF strain. The obtained complex contains primarily 50S ribosomal subunits (90%). With this complex two local reconstructions were obtained at 2.54 Å and 3.36Å, respectively The model reveals several interaction surfaces with ribosomal proteins and RNA, most notably through the a stretch of Lys in the helical domain.

On this background the authors then embark on a more detailed structural and cell biology investigation of the role of YchF/Ola1. Ultimately the authors report in E. coli and Yeast that the helical domain is essential for YchF function which has been reported before and that the H2O2 phenotype can be complement with the WT enzyme. Based on the identified binding site on the ribosome then authors speculate that Ychf might be able to split 70S ribosomes and report increased subunit levels in a density gradient assay. Following this the authors investigate the function of Ola1 in HeLa cells and perform Ribo-seq. The respective analyses identify 297 genes that were upregulated and 254 genes that were down regulated, which the authors claim seems to be consistent with previous reported effects of Ola1/YchF e.g under H2O2 exposure/stress. The authors attribute this effect to a role of Ola1/YchF on pausing of ribosomes translating D/E rich proteins.

Major concerns.

1.) The reported structure is based on a low populated state of YchF on the ribosome that is not consistent with previous reported cross-lining data (e.g. Landwehr et al., 2021). Together with the fact that the pull-down complex was stabilized by glutaraldehyde crosslinking this could suggest that it reflects an off-path complex/unspecific complex. The latter would be consistent with the highly unspecific mode of interaction with the ribosome (e.g. the Lys motif in the helical domain).

2.) Further, if the function of the Ola1/YchF is indeed ribosome splitting, in for example the translation of D/E rich proteins, this reported complex represents likely the post splitting complex. What is the functional relevant pre-splitting complex?

3.) The splitting activity is only moderate and indirect in the reported assay and many other groups have used, for example, rapid kinetics/light scattering to demonstrate the splitting activity. As this is one of the key findings / parts of the discussion a biochemical characterisation of this indeed novel activity should be included.

4.) The structural dynamics of Ola1/YchF compares the relative position of domains from the highly dynamic YchF structure reported here with high resolution structures from other organisms (e.g H. influenzae). The observed changes in orientation of the domains are used as an argument to support the interaction of the YchF with the 50S subunit reported here but might simply be explained by the interpretation of the authors of the overall very dynamic structure of YchF in solution and on the 50S ribosome following stabilisation by glutaraldehyde cross linking during sample preparation.

In summary: The authors, based on a newly reported complex of the translational GTP/ATPase YchF/Ola1, propose a functional role for the regulation during translation pausing. The claimed regulation mechanism of Ola1 (in the title) is not provided / fully supported by the reported initial functional characterisation.

Reviewer #2

(Remarks to the Author)
This manuscript describes a cryo-EM study of an ATPase enzyme YchF bound to E. coli 50S subunit and functional studies of YchF and its eukaryotic counterpart Ola1 in yeast and human cells. The authors report (based on high-resolution cryo-EM analyses and polysome assays) that YchF binds to a specific region on the 50S subunit and catalyzes the splitting of 70S ribosomes into subunits. Next, the authors show that the a-helical domain – binding to the 50S – is critical in both YchF and eukaryotic Ola1 for oxidative stress adaptation in E. coli and yeast, supporting the findings of the structural analyses. Then the authors perform RNA-seq and Ribo-seq of WT and Delta-Ola1 human cells, revealing that Ola1 deletion results in a gene expression change, and appears to upregulate ribosome stalling on sequences encoding negatively charged amino acids. Overall, this work advances our understanding of a poorly characterized protein YchF/Ola1 and uncovers new aspects of translational regulation by this protein. The authors, however, can improve the discussion of the structural mechanism of YchF function revealed by their structural and biochemical results (see comments below). I recommend the publication of this manuscript after minor revision.

1. The “S1, S2, S3” nomenclature is confusing because it has canonically been used for small-subunit ribosomal proteins (and is still being used in the literature). Remove these from the figures and text (or replace with a clearer nomenclature, such as “site 1”).
2. Regarding sub-classification into 2.5 and 3.4 A maps:
a. The positions or conformations of YchF in these two reconstructions are different, according to the statement that there is “movement of the protein”. Please describe the structural differences because they provide insights into the structural dynamics of YchF on the 50S subunit. For example, these may occur along the trajectory of YchF dissociation from the subunit.
b. Make the sentence regarding protein occupancy clearer, for example: “Both reconstructions feature strong density for YchF(H114A), indicating that the weak density in the combined reconstruction is due to the movement of the protein rather than to sub-stoichiometric occupancy”.

3. Cryo-EM density quality for YchF is lower than for the rest of the 50S subunit and does not allow the placement of most side chains. Nevertheless, the density is sufficient for the placement of secondary structure. This must be reflected in the text, for example: “Because YchF binds at the 50S periphery, the local resolution is lower than the average map resolution (4-7 Å), yet the density allows to model the secondary structure of YchF (Extended Fig. X).”

4. Panels d,e,f in figure 2 show strange patches of density for selected residues. It is a good idea to show local density, but density should cover the whole model in the view rather than carved around hand-picked residues. Update these panels so that continuous density is shown at the same contour level.

5. The presence of the TGS domain in YchF is curious. A TGS domain is also present in the stringent factor RelA, which senses deacyl-tRNA on 70S ribosomes. Cryo-EM structures found that the TGS domain binds the tRNA and 30S subunit ((see these three studies: https://www.nature.com/articles/nature17675;
https://pubmed.ncbi.nlm.nih.gov/27226493/;
https://elifesciences.org/articles/17029).
How does the TGS domain in YchF compare with that of RelA? Could it interact with a similar region of the 30S subunit as RelA? I suggest that the authors discuss this similarity with RelA, along with the potential roles of the TGS domain in YchF.

6. The observation of 70S splitting with either ATP or ADPNP is important. Expand the discussion that ATPase activity is not required for ribosomal subunit dissociation. This suggests that ATP hydrolysis is likely required for YchF dissociation from the 50S rather than for ribosome splitting.

7. In comparisons of “free” and 50S-bound protein, what can the alignment help to explain? One possibility is that the protein needs to rearrange to be able to bind to the 50S or dissociate from the 50S. Is the "free" conformation incompatible with the 50S due to steric clashes? Or is there another reason for YchF to rearrange upon 50S binding? Discuss these mechanistic implications.

8. What is the rationale for aligning on the helical domain rather than on other domains? If ATP hydrolysis induces a

conformational - and functionally relevant - change of the protein, wouldn't the alignment on the ATPase domains be a better idea to reveal the relative movements of other domains? For example, comparing ATP- and ADP-bound structures could explain the mechanism of ATPase-dependent dissociation of YchF from the 50S.

9. From the 50S structure description, the mechanism of 70S splitting by YchF remains unclear? Does YchF on the 50S overlap with the binding site for 30S, so that it shifts the equilibrium (70S<-> 50S+30S) toward dissociation. This important mechanistic conclusion needs to be discussed.

10. Experiments shown if Fig. 3c and Fig 3d must be (a) done in replicates (at least n=3) and (b) quantified. For example, plot/tabulate the disc area/radius.

11. It is unclear how similar/distant the bacterial and eukaryotic YchF/Ola1 homologs are. I suggest adding a figure/panel showing sequence alignment, and structure alignment if the eukaryotic structure is available.

12. To better explain the rationale for RNA-seq and Ribo-seq, I suggest that the sentence “Next, cells with 80% confluency… and sent for Ribo-seq analysis” is changed to “… and sent for RNA-seq and Ribo-seq analyses to characterize the changes in mRNA abundance and translation”.

13. In this and other sentences where applicable (e.g. “ Results showed that in Δola1 cells, 375 genes were up regulated…”), be more specific regarding methods or results. For example, this sentence could be “RNA-seq data showed that..,”. Also, by “vessel development”, do you mean “blood vessel development”?

14. Replace “coil-coil” with “coiled coil” or with “alpha4-alpha5 coiled coil” for clarity (using the Greek “alpha”).

15. In Table S1, report the structure’s correlation coefficient against the maps (CC; global, also report local CC for YchF). In addition, report the statistics for the RNA model (backbone outliers etc). In the Ramachandran plot, are "Outliers" in the allowed or disallowed region?

16. In Ext. Fig. 6, what are the units shown on the electrostatic potential heatmap? These numbers (around 80) look strange, as they are unlikely to correspond to the local charge units (which are usually on the order of 1-2 electrons/protons).

17. Proofread the manuscript to correct numerous typos and stylistic errors – including Methods (e.g. “Holley” and many more typos).

Andrei Korostelev

Reviewer #3

(Remarks to the Author)
This paper uses a suite of experimental approaches to uncover the molecular workings of Ola1/YchF Obg-Like GTPases from E. coli, yeast (unclear the species; I assume it is S. cerevisiae) and H. sapiens. These factors are well-known to be involved in resistance to stress, but the biological function is less clear, although both bacterial and eukaryotic factor were earlier implicated in translation, and direct interaction with the ribosome was shown. The authors use a melange of approaches: cryoEM (in E. coli), RNA-Seq and Ribo-Seq (in HeLa), phenotypic assays (different in different experimental systems) and molecular biology (ribosome splitting assays using yeast lysates). The result of this effort is rather confusing, with the individual parts being somewhat disconnected from each other. The paper reads like three papers assembled sequentially and this collection would be well-suited for a special issue on this protein family in a specialised journal. I have several specific suggestions:

- The three proteins studied in the three experimental systems are all from the Obg-Like protein family. However, it is unclear if these proteins have the same exact function in eukaryotes and bacteria. Therefore, the key results from different systems should be cross-validated between the experimental systems and followed up with orthogonal methods. Stalling on D/E-rich regions observed in HeLa lacking Ola1 should be i) validated in yeast and bacteria ii) tested using stalling reporters.
- Currently the yeast data does not add much. Validating the structural data obtained with E. coli in E. coli system would make much more sense. Polysome profiles, genetic interactions with other ribosome rescue systems, stalling reporters are the assays one would use.
- The connections between the mechanism and the phenotypes are unclear and confuse the reader further.
- The word ‘regulate’ is misused pervasively. The function of the factor seems to be resolving ribosomal stalling. Loss of the factor through genetic disruption is not ‘regulation’ as the authors do not demonstrate the existence of a specific response that regulates the expression level / activity of the factor in response to ribosomal stalling.
- Introducing the relevant literature on ribosomal stalling and the multiple factors that rescue stalled ribosomes is essential. In general the Introduction is exceedingly short.
- Nomenclature: ΔychF in bacteria but ola1Δ in yeast (note the positioning of Δ). In human cell lines no Δ is used; homozygous KO should be referred to as superscripted –/–, and the gene name should be capitalised.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript “Conserved GTPase OLA1 promotes efficient translation on D/E-rich mRNA” reports the structure of the highly conserved GTPase Ola1/YchF, which is a member of the TRAFAC (translation factors) class of GTPases. The molecular mechanism and cellular function of Ola1 and its bacterial homolog YchF have been elusive for decades but mounting biochemical evidence indicates a ribosome associated role. Recent biochemical studies using UV inducible crosslinkers locate Ola1/ YchF to the E-site on the 30S small ribosomal subunit. The work reported in this manuscript uses affinity purification of a his-tagged catalytically inactive variant to enrich for YchF-Ribosome complexes. Initial biochemical studies by the authors indicate low complex occupancy which they seek to overcome by the use of the inactive variant for enrichment and subsequent glutaraldehyde crosslinking to stabilize the enriched YchF-50S ribosomal complex. The resulting structure provides the first direct visualisation of YchF on the ribosome and locates it close to the A-site in the 50S ribosomal subunit.

Based in the locations the authors hypothesise a potential function in ribosome splitting and/or reassociation prevention. The authors attempt to support this by a reconstituted splitting experiment followed by density gradient centrifugation, in which they see an increased ration of 50S to 70S, which the authors claim suggests splitting (disassembly of the 70S ribosomes into the constituting the 30S and 50S subunits). However, the experiment fails to report 30S subunits (which is easily obtainable using density gradient centrifugation) and the authors therefore do not correlate the changed 50S:70S ratio with an expected proportional increase in the 30S:70S ratio, maintaining a 1 to1 30S:50S ratio. Since no additional biochemical (e.g. kinetic observation of splitting or inhibition of re-association of the subunits) is provided to supplement this, the data does not support the claim of a splitting or anti-association activity of YchF.

To investigate the function of Ola1/YchF the authors then switch from the bacterial system to Eukaryotes and perform mRNA-seq in HeLa cells. The respective analyses identify several differentially regulated genes which the authors claim seems to be consistent with previous reported effects of Ola1/YchF. Using Ribo-seq the authors then identified a role of OLA1/YchF in the translation of D/E-rich mRNA.

The flow of the MS has significantly improved but this reviewer is still confused about the importance for the section “Deletion of OLA1 increases the migration rate of Hela cells”

Reviewer #2

(Remarks to the Author)
The authors have most of my concerns, and the revised manuscript reads better than the initial submission. Several issues, however, remain to be addressed:
1. The authors claim that the buried surface area of 643 A^2 represents “a large interface among protein complexes”. They refer to a study presenting a machine-learning-based model for predicting binding affinities from buried surface areas. It is unclear how this work is relevant; perhaps the authors used this model to predict the affinity of YchF – which they should clarify. Nevertheless, if compared with other protein complexes, the BSA of ~650 A^2 would be on the lower end of binding affinities (as complexes generally have the BSAs of 500 to 2000, and higher than 2000 for very stable complexes) - https://onlinelibrary.wiley.com/doi/10.1002/pro.2230
2. In the section describing the helical domain of YchF, the authors state that “YchF is a multifunctional protein”. The molecular functions of YchF, as described in the introduction, are primarily linked to translation and depend on ribosome binding. In this work, the authors propose that they have elucidated the molecular mechanisms of YchF function, which is to to bind to the large subunit to promote ribosome dissociation. What other functions of “multifunctional YchF” do the authors refer to in this section? They need to elaborate on that and explain the importance of the 50S binding function relative to other functions.
3. In Supp. Fig. 9, the authors show that YchF fails to split the 70S, by incubating the ribosomes with the “5-fold excess” of YchF. Given that the binding affinity is likely low, have they tried a much higher excess (e.g. 100-fold)? This would support their conclusion that YchF is indeed an anti-association factor.
4. The description of the experiment mCherry-D12-GFP experiment is misleading and the data in human cells do not support the conclusion that OLA1 affects the D12 reporter: the is no difference in GFP/mCherry between WT and OLA1-/- cells (Fig. 6a-c).
5. In Fig. 2, it is unclear which species were used for sequence alignment and WebLogo. It is not clear how the species were selected for multiple sequence alignments in this work. This needs to be clarified in figure legends and Methods.
6. In Methods, what was the complex concentration in the 3 uL sample aliquots applied to cryo-EM grids?
7. The manuscript, including Methods and other sections, needs to be further carefully proofread (e.g. “Laplacin-of-Guassin”, “palys” and more).

Andrei Korostelev

Reviewer #3

(Remarks to the Author)
I am impressed with the effort the authors have put into revision. The paper is now much improved.

When it comes to ribosomal stalling on charged motifs - and cellular factors that can assist the ribosome in negotiating these - there are a couple of recent papers are relevant and worth citing:

Resolution of ribosomal stalling by EF-P and ABCF ATPases YfmR and YkpA/YbiT
Hiraku Takada, Keigo Fujiwara, Gemma C Atkinson, Shinobu Chiba, Vasili Hauryliuk
Nucleic Acids Research, Volume 52, Issue 16, 9 September 2024, Pages 9854–9866,

The ABCF proteins in Escherichia coli individually cope with 'hard-to-translate' nascent peptide sequences Yuhei Chadani, Shun Yamanouchi, Eri Uemura, Kohei Yamasaki, Tatsuya Niwa, Toma Ikeda, Miku Kurihara, Wataru Iwasaki, Hideki Taguchi
Nucleic Acids Research, Volume 52, Issue 10, 10 June 2024, Pages 5825–5840

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
After confirming the splitting activity of YchF, the only comment is that the authors should be clear about the fact that the 50S complex likely resembles the post splitting complex and that this might be different than the 70S encounter complex.

Besides this my main concerns have been addressed.

Reviewer #2

(Remarks to the Author)
The authors addressed most of my comments and improved the manuscript. It is great to see that additional experiments have been performed and contributed to this work. Two inconsistencies, however, still need to be addressed to ensure the study is rigorous.
1. The BSA of 643 A^2 usually (but not always, as authors correctly note in their response) indicates weak to modest binding between macromolecules. The authors modified their conclusion to "This accounts for a reasonable interface among protein complexes30,31, suggesting a stable binding of YchF to the 50S subunit."

Based on their own measurements of YchF action on 70S dissociation, however, they concluded that "Lower amount (5-fold excess) of YchF did not increase the splitting of 70S. These results indicated that YchF binds weakly to the purified ribosomes or subunits, which is consistent with the binding assay shown in Extended Data Fig. 3a."

This inconsistency is easy to resolve by interpreting the BSA calculations as "This accounts for a lower to modest interface among protein complexes 30,31, suggesting a weak or modest binding consistent with our experimental data discussed below".

2. The response to my point about Fig. 6c is inadequate: the data for human cells do not support the conclusion that OLA1 affects the D12 reporter because the difference between WT and OLA-/- is very small and the spread overlaps. The authors should clearly point out that the effect of OLA1 in human cells was not as conclusive as for Ychf in E. coli cells, possibly due to the complex regulatory mechanisms in human cells.

3. Proofreading of the manuscript is still necessary, as numerous typos and stylistic errors must be fixed. Another suggestion is to use the present tense for new findings instead of the past tense (e.g. "Here, we reported …").

Andrei Korostelev

Reviewer #1 (Remarks to the Author):

The manuscript “Structure and regulation mechanism of Ola1 on the translation pausing” reports the structure of the highly conserved GTPase Ola1, which is a member of the TRAFAC (translation factors) class of GTPases. The molecular mechanism and cellular function of Ola1 and its bacterial homolog YchF have been a mystery since its discovery several decades ago. Many studies have reported critical roles in various cellular stress responses, and it has been implicated in the regulation of tumor progression in numerous types of cancers in Eukaryotes. Many studies have indicated a ribosome associated role and although structural information for the protein is available form a wide range of organisms, a complex bound to the ribosome is still missing. Recent biochemical studies using UV inducible crosslinkers indeed support binding of the YchF/Ola1 to the ribosome, crosslinks primary mapping to the E-site on the 30S subunit.

Here the authors report the structure of E. coli YchF bound to the 50S ribosomal subunit as determined by cryo-electron microscopy. This structure places the YchF into the A-site of the 50S subunit close to the translation-factor (EF-Tu / EF-G) binding site. To obtain this complex the authors used several different conditions including different non-hydrolysable analogs of ATP and GTP. Previous work has shown that Ola1/YchF although member of the GTPase family has a drastically altered Nucleotide binding pocket resulting in the different specificities for GTP and ATP depending on the organism. On this background the authors observed only weak binding to the ribosome (< 30% to the 70S ribosome) when in vitro reconstructing the complexes. The authors then proceed to directly purify the ribosome complex using the His-Tagged version of a previously reported hydrolysis inactive variant of the YchF (YchF H114A) form the delta YchF strain. The obtained complex contains primarily 50S ribosomal subunits (90%). With this complex two local reconstructions were obtained at 2.54 Å and 3.36Å, respectively The model reveals several interaction surfaces with ribosomal proteins and RNA, most notably through the a stretch of Lys in the helical domain.

On this background the authors then embark on a more detailed structural and cell biology investigation of the role of YchF/Ola1. Ultimately the authors report in E. coli and Yeast that the helical domain is essential for YchF function which has been reported before and that the H2O2 phenotype can be complement with the WT enzyme. Based on the identified binding site on the ribosome then authors speculate that Ychf might be able to split 70S ribosomes and report increased subunit levels in a density gradient assay. Following this the authors investigate the function of Ola1 in HeLa cells and perform Ribo-seq. The respective analyses identify 297 genes that were upregulated and 254 genes that were down regulated, which the authors claim seems to be consistent with previous reported effects of Ola1/YchF e.g under H2O2 exposure/stress. The authors attribute this effect to a role of Ola1/YchF on pausing of ribosomes translating D/E rich proteins.

Major concerns.

1.)The reported structure is based on a low populated state of YchF on the ribosome that is not consistent with previous reported cross-lining data (e.g. Landwehr et al., 2021). Together with the fact that the pull-down complex was stabilized by glutaraldehyde crosslinking this could suggest that it reflects an off-path complex/unspecific complex. The latter would be consistent with the highly unspecific mode of interaction with

the ribosome (e.g. the Lys motif in the helical domain).

Response: Thank you for the comments. We used glutaraldehyde crosslinking mainly because many ribosomal complexes are unstable and tend to dissociate during cryo-EM sample preparation. Prior to this, we attempted extensive purification but were unable to obtain the corresponding complexes. This is quite a common issue: although biochemical methods confirm complex formation, cryo-EM often shows empty ribosomes. For example, in our study of the Rbg1/Tma46 complex, it was only after applying glutaraldehyde crosslinking that we were able to obtain the structure of the Rbg1/Tma46-bound ribosome following cryo-EM sample preparation (reference #1). In the study by Pochopien, A.A. et al. on Gcn1, glutaraldehyde crosslinking was also used to stabilize the complexes (reference #2).

We believe that the structure of the YchF/50S complex obtained in our study is specific based on the following points:

1. After pull-down, we first used 1 M sucrose cushion to remove proteins that were not bound to the ribosome. After removing the free proteins, even some unspecific binding exists, some free 50S should be observed in the cryoEM structure. However, nearly all 50S subunits are bound to YchF, and only specific pull-down could achieve such a high proportion of complexes.
2. Based on our laboratory's experience, if the pull-down is non-specific, only a small amount of ribosomes can be obtained, and the proportion of 70S would be higher. However, as our results indicate, 90% of the particles are 50S, which suggests a specific interaction.
3. The binding of YchF to the 50S subunit primarily involves three sites. At site 1, the arginine interacts with rRNA, largely through electrostatic interactions. Sites 2 and 3 involve additional potential hydrogen bonding interactions and display shape complementarity, a key characteristic of protein binding interfaces. Additionally, the area of these interfaces was calculated as more than 643 $Å^2$, accounting for a large interface among protein complexes, indicating a stable binding of YchF with 50S subunit (Please see results part #2 for this statement).
4. In our revised Fig.3, we compared the conformational changes between free and 50S-associated YchF and represented the shift of α7 in ATPase domain. The α7 in free state has clash with uL14, which means it can not directly bind to 50S. If the binding is a result from crosslinking, the sucrose cushion before crosslinking will remove all the free protein.

Based on this analysis, we are inclined to believe that the binding of YchF to the 50S subunit is specific.

Reference #1: Zeng, F., Li, X., Pires-Alves, M., Chen, X., Hawk, C.W. and Jin, H. (2021) Conserved heterodimeric GTPase Rbg1/Tma46 promotes efficient translation in eukaryotic cells. Cell Rep, 37, 109877.

Reference #2: Pochopien, A.A., Beckert, B., Kasvandik, S., Berninghausen, O., Beckmann, R., Tenson, T. and Wilson, D.N. (2021) Structure of Gcn1 bound to stalled and colliding 80S ribosomes. Proc Natl Acad Sci U S A, 118.

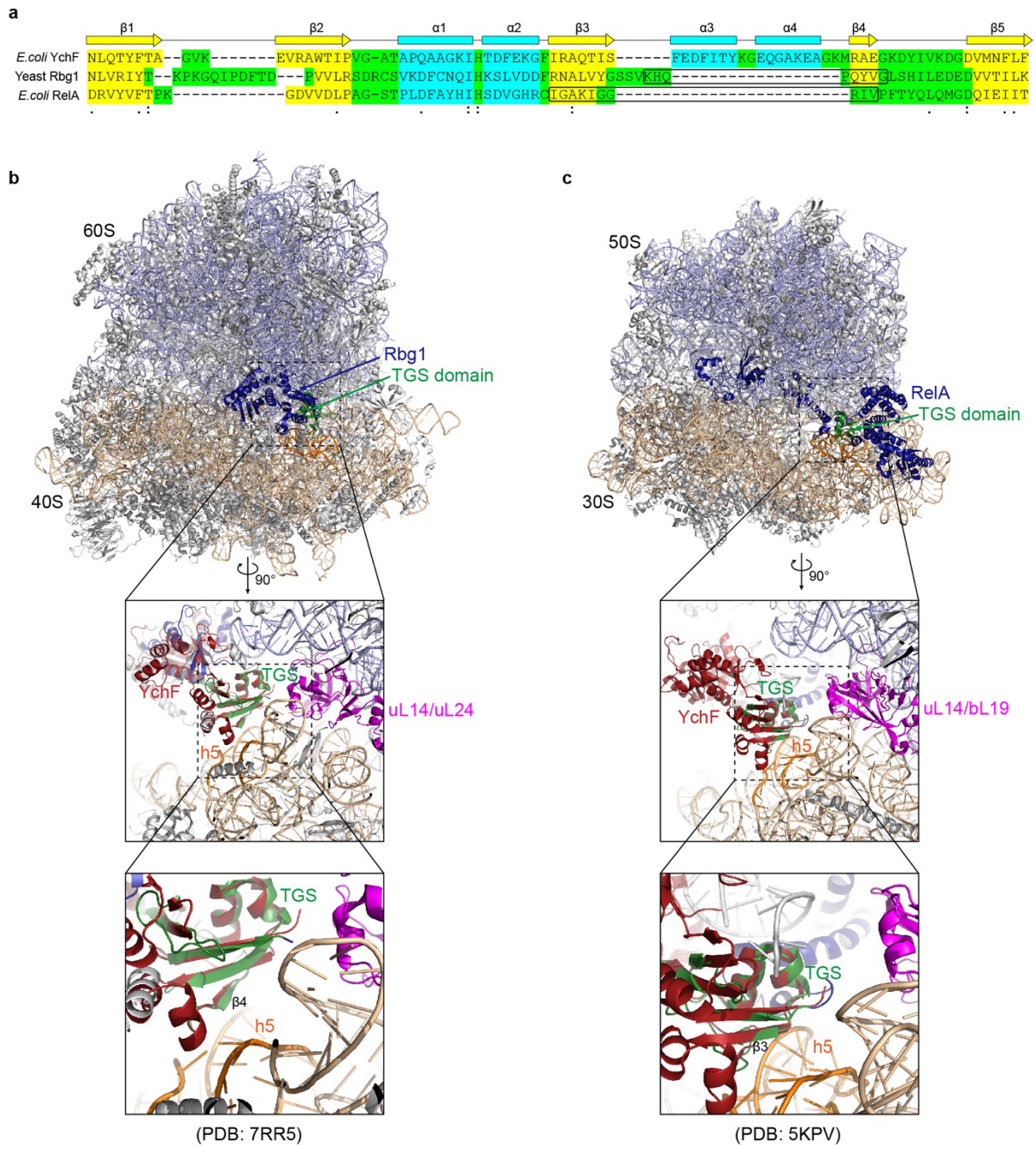
2.)Further, if the function of the Ola1/YchF is indeed ribosome splitting, in for example the translation of D/E rich proteins, this reported complex represents likely the post splitting complex. What is the functional relevant pre-splitting complex?

Response: Thank you for the question. Yes, the observed complex represents a post-splitting state. In our revised manuscript, we have compared the TGS domain between YchF, RelA, and Rbg1 as suggested by reviewer #2. The TGS domain in both RelA and Rbg1 could interact with rRNA h5 of the 30S subunit, and h5 is close to uL14/bL19 (the binding site of YchF in our study) on 70S, suggesting the possibility that YchF also binds to h5 via its TGS domain. We hypothesize that YchF might initially bind to the 70S via its TGS domain, possibly in conjunction with other ribosome-associated factors like Pth. Subsequently, through certain changes, the 50S and 30S subunits may be splitted, with YchF remaining on the 50S to prevent further assembly with the 30S. This process resembles how, after the RQT complex separates the 60S from 40S, eIF6 binds to the 60S, preventing the formation of 80S.

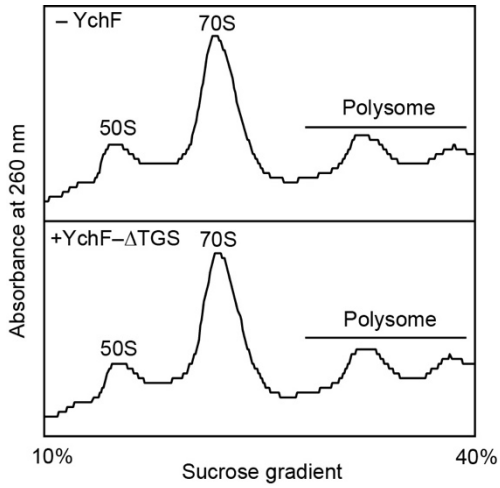
Discussion has been added to the revised manuscript:

"The reported complex in our study represents likely the post splitting complex. How does OLA1/YchF recognize the stalled ribosome and promote the splitting? We noticed that the TGS domains of Rbg1 and RelA both could interact with h5 of the 18S/16S rRNA on the 80S/70S ribosome[25,42] (Extended Data Fig. 15). Structure based alignment of TGS domain in YchF, Rbg1 and RelA showed that their sequences are not conserved but structures are similar (Extended Data Fig. 15a). Rbg1 uses the loop after β3 and partial β4 to interact with h5 (Extended Data Fig. 15b). RelA adopts similar pattern but including β3 for the interaction (Extended Data Fig. 15c). Superimposing of the TGS domain on the ribosome showed that YchF may also be able to bind to this region. Since h5 is close to the uL14/bL19, which is the binding site of YchF in 50S subunit. We hypothesized that OLA1/YchF might initially bind to the stalled ribosome via its TGS domain, possibly in conjunction with other ribosome-associated factors like Pth. Subsequently, through certain changes, the large and small subunits may be split, with OLA1/YchF remaining on the large subunit to prevent re-association with the small subunit. This process resembles how, after the RQC trigger complex (RQT) separates the 60S from 40S, eIF6 binds to the 60S, preventing the formation of 80S[26]."

The two figures that support this hypothesis are shown below:



Extended Data Fig. 15 | YchF might target to 70S by TGS domain. a, Sequence alignment of the TGS domain of *E.coli* YchF, *S.cerevisiae* Rbg1 and *E.coli* RelA. The sequence alignment was based on the results of the structural alignment. Helix, α-strand and loop are colored cyan, yellow and green, respectively. The binding sites of TGS to the h5 of 16S rRNA are marked with black boxes. The symbol '.' and ':' indicates the similar and identical amino acids, respectively. **b**, **c**, Superimposing YchF on Rbg1 (**b**) and RelA (**c**) based on the TGS domains. Rbg1 and RelA are shown in blue with TGS domain in green, while YchF is depicted in red. The binding site of YchF on 50S in this study are colored magenta. For clarity, the hydrolase (HYD) and synthetase (SYN) domains of RelA have been hidden in the enlarged subfigure (**c**).



Extended Data Fig. 16 | Deletion of TGS domain omits the splitting activity of YchF. Polysome profiling of Δ*ychF* cells with the treatment of chloramphenicol in the absence (upper) or precence of 5 μM YchF-ΔTGS with 2 mM of ATP.

3.)The splitting activity is only moderate and indirect in the reported assay and many other groups have used, for example, rapid kinetics/light scattering to demonstrate the splitting activity. As this is one of the key findings / parts of the discussion a biochemical characterisation of this indeed novel activity should be included.

Response: Thank you for your suggestion. Indeed, a direct splitting assay would fully demonstrate the activity of OLA1/YchF. However, this is hard to be done at this point since:

1. These assays need a pure stalling 70S complex on D/E-rich mRNA. With current techniques, we are unable to purify this complex. Even for the R/K-rich mRNA, after years of attempts by our group and many others, the target complex has not yet been successfully purified. Purifying these complexes is crucial for answering many questions about the translation regulation process, so we will continue to explore the necessary conditions.
2. We have not yet identified which proteins are involved in the process of YchF promoting the splitting of stalled 70S ribosomes on D/E-rich mRNA. Even if we can purify stalled ribosomes on D/E-rich mRNA, we currently lack the capability to test for splitting activity.

To verify the importance of OLA1/YchF on D/E-rich mRNA, we constructed the mCherry-D12-GFP reporter system and measured the GFP/mCherry ratio using flow cytometry (results part #7 in the revised manuscript). The results indicate that deletion of OLA1/YchF significantly impacts the translation of these two proteins (shown below, which was merged with Fig.6 in the revised manuscript).

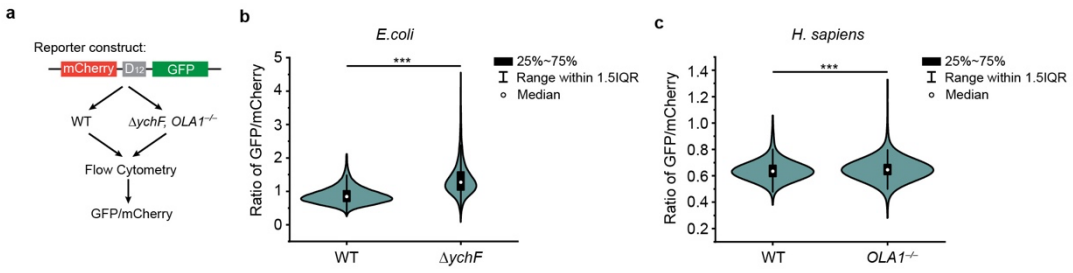


Figure b and c showed that deletion of OLA1/YchF causes an increasing of GFP/mCherry, which can be interpreted as the ribosome quality control (RQC) pathway being activated, leading to the degradation of the mCherry protein.

4.)The structural dynamics of Ola1/YchF compares the relative position of domains from the highly dynamic YchF structure reported here with high resolution structures from other organisms (e.g H. influenzae). The observed changes in orientation of the domains are used as an argument to support the interaction of the YchF with the 50S subunit reported here but might simply be explained by the interpretation of the authors of the overall very dynamic structure of YchF in solution and on the 50S ribosome following stabilisation by glutaraldehyde cross linking during sample preparation.

Response: Thank you for this important observation. We acknowledge that the structural dynamics of YchF/Ola1, including the changes in domain orientation, may be interpreted differently. In our revised Fig.3, we compared the conformational changes between free and 50S-associated YchF and represented the shift of α7 in ATPase domain. The α7 in free state has clash with uL14, which means it can not directly bind to 50S. This differences in conformation could simply be due to binding on the ribosome, or they may reflect conformational variations of OLA1/YchF in different nucleotide states. The impact from glutaraldehyde cross linking would be minimum as discussed in the Comment #1.

Discussion about these conformational changes has been add to the Results part 3 in the revised manuscript.

"Since the helical domain is the main binding site to the 50S (Fig.2), we aligned the helical domain from different species. Our alignment results reflected the dramatic shifts and rotations in ATPase and TGS domains (Extended Data Fig. 7d). For instance, shifts in α7 helix of ATPase domain towards uL14, causing a steric clash with uL14 protein (Fig. 3a). This shift of α7 is primarily due to the conformation changes between the free state and the 50S-associated state of YchF (Extended Data Fig. 7d). …… Alignment results further suggested smallest cleft angle for YchF on the ribosome, which showed a proper conformation of α7 for the binding of protein to 50S. The differences in conformation could be due to binding on the ribosome, or they may reflect conformational variations of OLA1/YchF in different nucleotide states. However, it is challenging to determine the specific causes without obtaining distinct structures of YchF from *E.coli* in various states."

In summary: The authors, based on a newly reported complex of the translational GTP/ATPase YchF/Ola1, propose a functional role for the regulation during translation pausing. The claimed regulation mechanism of Ola1 (in the title) is not provided / fully supported by the reported initial functional characterisation.

Response: Thank you for your comment. We acknowledge that the current data does not fully explain how the OLA1/YchF regulates the ribosome stalling on D/E-rich mRNA. In the revised manuscript, we have adjusted the title to 'Conserved GTPase OLA1 promotes efficient translation on D/E-rich mRNA'. Additionally, we have refined our discussion to more accurately reflect the extent of the functional characterization and avoid overstating conclusions that are beyond the scope of the present findings.

Reviewer #2 (Remarks to the Author):

This manuscript describes a cryo-EM study of an ATPase enzyme YchF bound to E. coli 50S subunit and functional studies of YchF and its eukaryotic counterpart Ola1 in yeast and human cells. The authors report (based on high-resolution cryo-EM analyses and polysome assays) that YchF binds to a specific region on the 50S subunit and catalyzes the splitting of 70S ribosomes into subunits. Next, the authors show that the a-helical domain – binding to the 50S – is critical in both YchF and eukaryotic Ola1 for oxidative stress adaptation in E. coli and yeast, supporting the findings of the structural analyses. Then the authors perform RNA-seq and Ribo-seq of WT and Delta-Ola1 human cells, revealing that Ola1 deletion results in a gene expression change, and appears to upregulate ribosome stalling on sequences encoding negatively charged amino acids. Overall, this work advances our understanding of a poorly characterized protein YchF/Ola1 and uncovers new aspects of translational regulation by this protein. The authors, however, can improve the discussion of the structural mechanism of YchF function revealed by their structural and biochemical results (see comments below). I recommend the publication of this manuscript after minor revision.

1.The “S1, S2, S3” nomenclature is confusing because it has canonically been used for small-subunit ribosomal proteins (and is still being used in the literature). Remove these from the figures and text (or replace with a clearer nomenclature, such as “site 1”).

Response: Thank you for the suggestion. In the revised manuscript, we have replaced ‘S1, S2, S3’ with ‘site 1, site 2, site 3’ to avoid confusion.

2.Regarding sub-classification into 2.5 and 3.4 A maps:

a. The positions or conformations of YchF in these two reconstructions are different, according to the statement that there is “movement of the protein". Please describe the structural differences because they provide insights into the structural dynamics of YchF on the 50S subunit. For example, these may occur along the trajectory of YchF dissociation from the subunit.

Response: Thank you for your suggestion. The two reconstructions only showed little difference in the ATPase domain and TGS domain (as shown in revised Ext. Fig. 4f). We believed this may simply be due to some flexibility in the positions of the TGS, ATPase, and Helical domains, as these domains are primarily connected by loops. The differences between them might not necessarily represent the trajectory of YchF dissociation from the subunit.

Description about this was added to the revised manuscript:

“By aligning the rRNA in the two local reconstructions, the helical domain showed identical conformations,

while ATPase and TGS domain represented a small shift (< 2 Å). This shift may be due to flexibility in the positions of the Helical, ATPase, and TGS domains, as these domains are primarily connected by loops."

b.Make the sentence regarding protein occupancy clearer, for example: "Both reconstructions feature strong density for YchF(H114A), indicating that the weak density in the combined reconstruction is due to the movement of the protein rather than to sub-stoichiometric occupancy".

Response: Thank you for your suggestion. This sentence and the one in next comment have been revised (see Response for Comment #3).

3. Cryo-EM density quality for YchF is lower than for the rest of the 50S subunit and does not allow the placement of most side chains. Nevertheless, the density is sufficient for the placement of secondary structure. This must be reflected in the text, for example: "Because YchF binds at the 50S periphery, the local resolution is lower than the average map resolution (4-7 Å), yet the density allows to model the secondary structure of YchF (Extended Fig. X)."

Response: Thank you for your suggestion. These two sentences (Comments #2 and #3) have been changed in the revised manuscript as shown below:

"Both reconstructions featured strong density for YchF(H114A), indicating that the weak density in the combined reconstruction is due to the movement of the protein rather than to sub-stoichiometric occupancy. Because YchF(H114A) binds at the 50S periphery, the local resolution is lower than the average map resolution (4-7 Å), yet the density allows to model the secondary structure of YchF(H114A) (Extended Data Fig. 5)."

4. Panels d,e,f in figure 2 show strange patches of density for selected residues. It is a good idea to show local density, but density should cover the whole model in the view rather than carved around hand-picked residues. Update these panels so that continuous density is shown at the same contour level.

Response: Thank you for your suggestion. Fig.2 has been revised and densities have covered the whole model in the view.

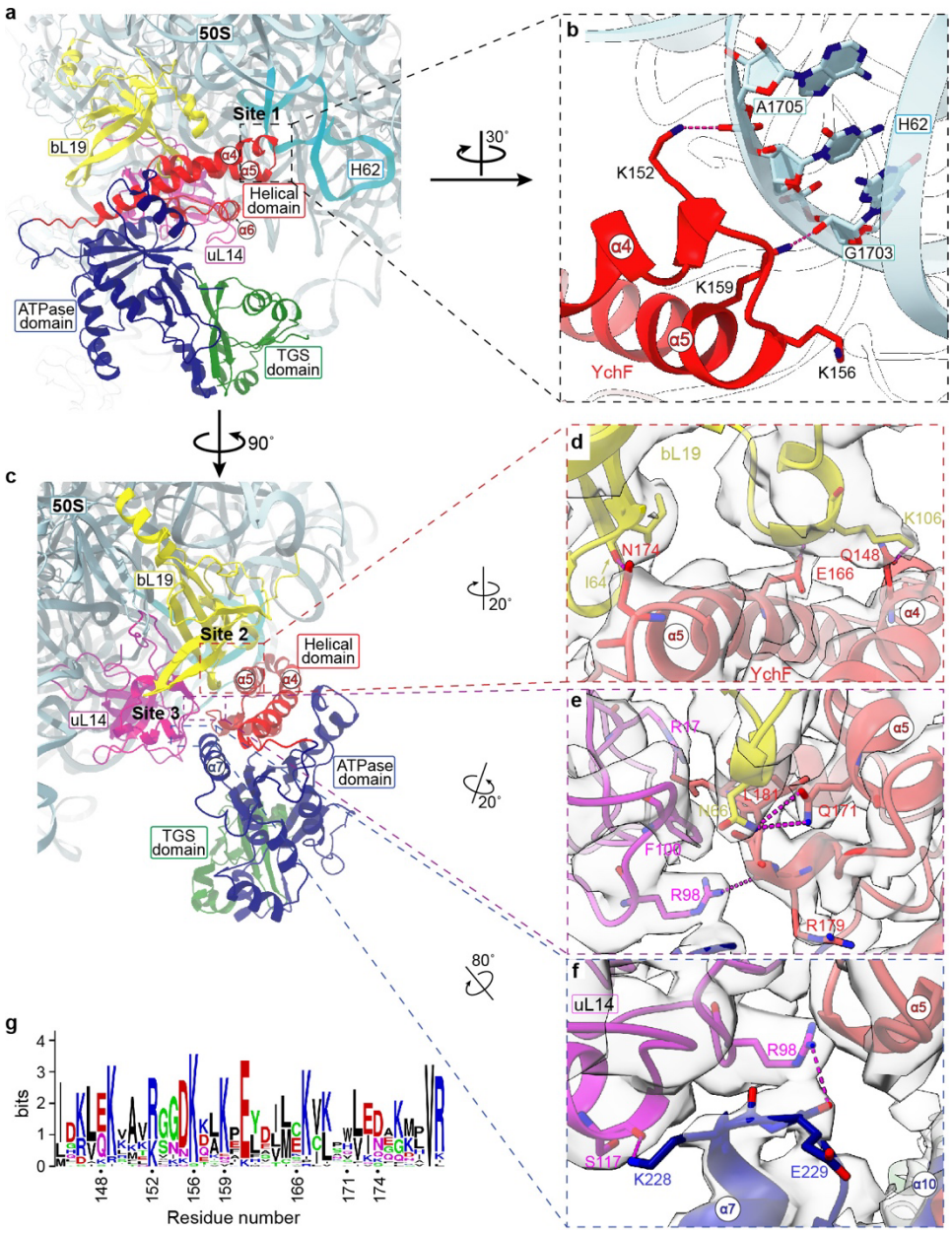


Fig. 2 | The detailed interactions between the YchF(H114A) and 50S subunit. a, c, Overview of the interactions between YchF(H114A) (red/blue/green) and 50S (uL14: magenta; bL19: yellow; H62: cyan) shown in two representative views. **b**, Magnified view of the region indicated by box in **a**.The key residues of YchF and H62 involved in YchF-50S interactions are shown and labelled. **d-f**, Magnified views of the regions indicated by boxes in **c**. The key residues of YchF and uL14, bL19 involved in YchF-50S interactions are shown and labelled. Cryo-EM map densities are shown in grey with the same level. **g**, Sequence logos created in WebLogo shows that lysine and arginine are conserved in the tip of the helical domain among species.

5. The presence of the TGS domain in YchF is curious. A TGS domain is also present in the stringent factor RelA, which senses deacyl-tRNA on 70S ribosomes. Cryo-EM structures found that the TGS domain binds the tRNA and 30S subunit ((see these three studies: https://www.nature.com/articles/nature17675; https://pubmed.ncbi.nlm.nih.gov/27226493/; https://elifesciences.org/articles/17029).
How does the TGS domain in YchF compare with that of RelA? Could it interact with a similar region of the 30S subunit as RelA? I suggest that the authors discuss this similarity with RelA, along with the potential roles of the TGS domain in YchF.

Response: Thank you for your suggestion. We compared the sequences and structures of TGS domain in RelA, Rbg1, and YchF. Interestingly, both RelA and Rbg1's TGS interact with h5 of 16S/18S rRNA (references #1, #2) (newly added Ext. Fig. 15). We hypothesized that YchF might initially bind to the 70S via its TGS domain, possibly in conjunction with other ribosome-associated factors like Pth. Subsequently, through certain changes, the 50S and 30S subunits may be split, with YchF remaining on the 50S to prevent further assembly with the 30S. This process resembles how, after the RQT complex separates the 60S from 40S, eIF6 binds to the 60S, preventing the formation of 80S (references #3).

The discussion of TGS domain has been added into the model part as below:

Reference #1: Zeng, F. et al. Conserved heterodimeric GTPase Rbg1/Tma46 promotes efficient translation in eukaryotic cells. *Cell Rep* 37, 109877 (2021).

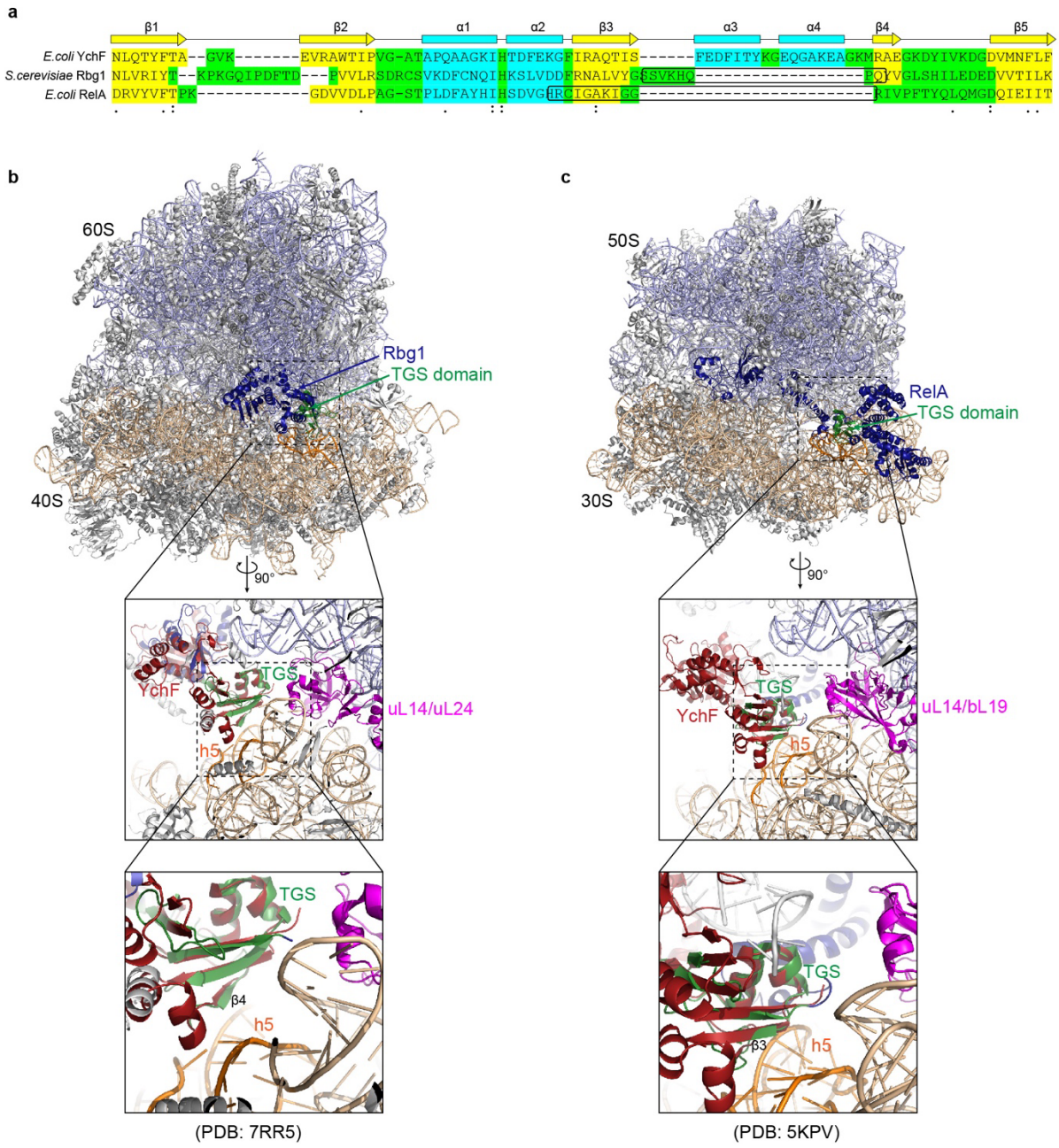
Reference #2: Loveland, A. B. et al. Ribosome*RelA structures reveal the mechanism of stringent response activation. *Elife* 5 (2016).

Reference #3: Best, K. et al. Structural basis for clearing of ribosome collisions by the RQT complex. *Nat Commun* 14, 921 (2023).

Description has been added to the revised manuscript:

"The reported complex in our study represents likely the post splitting complex. How does OLA1/YchF recognize the stalled ribosome and promote the splitting? We noticed that the TGS domains of Rbg1 and RelA both could interact with h5 of the 18S/16S rRNA on the 80S/70S ribosome[25,42] (Extended Data Fig. 15). Structure based alignment of TGS domain in YchF, Rbg1 and RelA showed that their sequences are not conserved but structures are similar (Extended Data Fig. 15a). Rbg1 uses the loop after β3 and partial β4 to interact with h5 (Extended Data Fig. 15b). RelA adopts similar pattern but including β3 for the interaction (Extended Data Fig. 15c). Superimposing of the TGS domain on the ribosome showed that YchF may also be able to bind to this region. Since h5 is close to the uL14/bL19, which is the binding site of YchF in 50S subunit. We hypothesized that OLA1/YchF might initially bind to the stalled ribosome via its TGS domain, possibly in conjunction with other ribosome-associated factors like Pth. Subsequently, through certain changes, the large and small subunits may be split, with OLA1/YchF remaining on the large subunit to prevent re-association with the small subunit. This process resembles how, after the RQC trigger complex (RQT) separates the 60S from 40S, eIF6 binds to the 60S, preventing the formation of 80S[26]."

And the Ext. Fig. 15:



Extended Data Fig. 15 | YchF might target to 70S by TGS domain. a, Sequence alignment of the TGS domain of *E.coli* YchF, *S.cerevisiae* Rbg1 and *E.coli* RelA. The sequence alignment was based on the results of the structural alignment. Helix, α-strand and loop are colored cyan, yellow and green, respectively. The binding sites of TGS to the h5 of 16S rRNA are marked with black boxes. The symbol '.' and ':' indicates the similar and identical amino acids, respectively. **b**, **c**, Superimposing YchF on Rbg1 (**b**) and RelA (**c**) based on the TGS domains. Rbg1 and RelA are shown in blue with TGS domain in green, while YchF is depicted in red. The binding site of YchF on 50S in this study are colored magenta. For clarity, the hydrolase (HYD) and synthetase (SYN) domains of RelA have been hidden in the enlarged subfigure (**c**).

6. The observation of 70S splitting with either ATP or ADPNP is important. Expand the discussion that ATPase activity is not required for ribosomal subunit dissociation. This suggests that ATP hydrolysis is likely required for YchF dissociation from the 50S rather than for ribosome splitting.

Response: Thank you for your suggestion. Discussion has been added to the revised manuscript as follows:

"Alignment of the TGS domain between YchF, Rbg1 and RelA suggested a possible binding site of OLA1/YchF on the stalled ribosome, which is h5 of the small subunit rRNA. We still do not know the details of the splitting process by lacking the structures of the pre-splitting complexes. However, based on the fact that YchF(H114A) binds to the 50S subunit, ATPase activity is most likely not required for ribosomal subunit dissociation, but important for YchF dissociation from the 50S subunit."

7.In comparisons of "free" and 50S-bound protein, what can the alignment help to explain? One possibility is that the protein needs to rearrange to be able to bind to the 50S or dissociate from the 50S. Is the "free" conformation incompatible with the 50S due to steric clashes? Or is there another reason for YchF to rearrange upon 50S binding? Discuss these mechanistic implications.

Response: Thank you for your suggestion. I'll response this one with the next comment.

8.What is the rationale for aligning on the helical domain rather than on other domains? If ATP hydrolysis induces a conformational - and functionally relevant - change of the protein, wouldn't the alignment on the ATPase domains be a better idea to reveal the relative movements of other domains? For example, comparing ATP- and ADP-bound structures could explain the mechanism of ATPase-dependent dissociation of YchF from the 50S.

Response: Thank you for your suggestion. We aligned the helical domain instead of ATPase domain because the binding site are mainly in helical domain. By this alignment, we showed that the "free" conformation is incompatible with the 50S due to steric clashes between α7 and uL14 (revised Fig. 3a). The alignment also showed that the ATP/ADP/free states could not give us clear conclusion. For example, the *O. sativa* YchF has the same conformation between apo protein and AMPPNP-bound states. The *T. thermophilus* YchF has similar conformation between apo and GDP-bound states. To discuss about the conformational changes between different nucleotide-bound states, we still need more structural information.

Description has been added to the revised manuscript:

"Since the helical domain is the main binding site to the 50S (Fig.2), we aligned the helical domain from different species. Our alignment results reflected the dramatic shifts and rotations in ATPase and TGS domains (Extended Data Fig. 7d). For instance, shifts in α7 helix of ATPase domain towards uL14, causing a steric clash with uL14 protein (Fig. 3a). This shift of α7 is primarily due to the conformation changes between the free state and the 50S-associated state of YchF (Extended Data Fig. 7d). …… Alignment results further suggested smallest cleft angle for YchF on the ribosome, which showed a proper conformation of α7 for the binding of protein to 50S. The differences in conformation could be due to binding on the ribosome, or they may reflect conformational variations of OLA1/YchF in different nucleotide states. However, it is challenging to determine the specific causes without obtaining distinct structures of YchF from *E.coli* in various states."

9.From the 50S structure description, the mechanism of 70S splitting by YchF remains unclear? Does YchF on the 50S overlap with the binding site for 30S, so that it shifts the equilibrium (70S<-> 50S+30S) toward dissociation. This important mechanistic conclusion needs to be discussed.

Response: Thank you for your suggestion. Yes, YchF on the 50S overlap with the binding site for 30S, it should be able to shift the equilibrium toward dissociation as observed in RsfS and eIF6, the two anti-association factors that bind to similar position as YchF does. However, according to our newly added results (Ext. Fig. 15 and 16), deletion of TGS domain omitted the splitting activity of YchF, indicating TGS may direct YchF to the stalled ribosome as what RelA and Rbg1 do. Discussion has been added as blew (see Response for comment #5 for details):

"…. We hypothesized that OLA1/YchF might initially bind to the stalled ribosome via its TGS domain, possibly in conjunction with other ribosome-associated factors like Pth. Subsequently, through certain changes, the large and small subunits may be split, with OLA1/YchF remaining on the large subunit to prevent re-association with the small subunit. This process resembles how, after the RQC trigger complex (RQT) separates the 60S from 40S, eIF6 binds to the 60S, preventing the formation of 80S[26]."

10.Experiments shown if Fig. 3c and Fig 3d must be (a) done in replicates (at least n=3) and (b) quantified. For example, plot/tabulate the disc area/radius.

Response: Thank you for your comments. We have conducted the experiments shown in Fig. 3b (original Fig. 3c) in replicates (n=4) and provided quantification in the revised manuscript (Fig. 3c) to ensure the robustness of the data and statistical analysis. The original Fig. 3d has been removed in the revised manuscript since yeast

data does not add much to the whole story. We removed all the yeast results and added more experiments in *E.coli* and Hela cells to validate our observation of YchF/OLA1 on promoting D/E-rich mRNA.

The revised Fig. 3b and 3c are shown below with their legends:

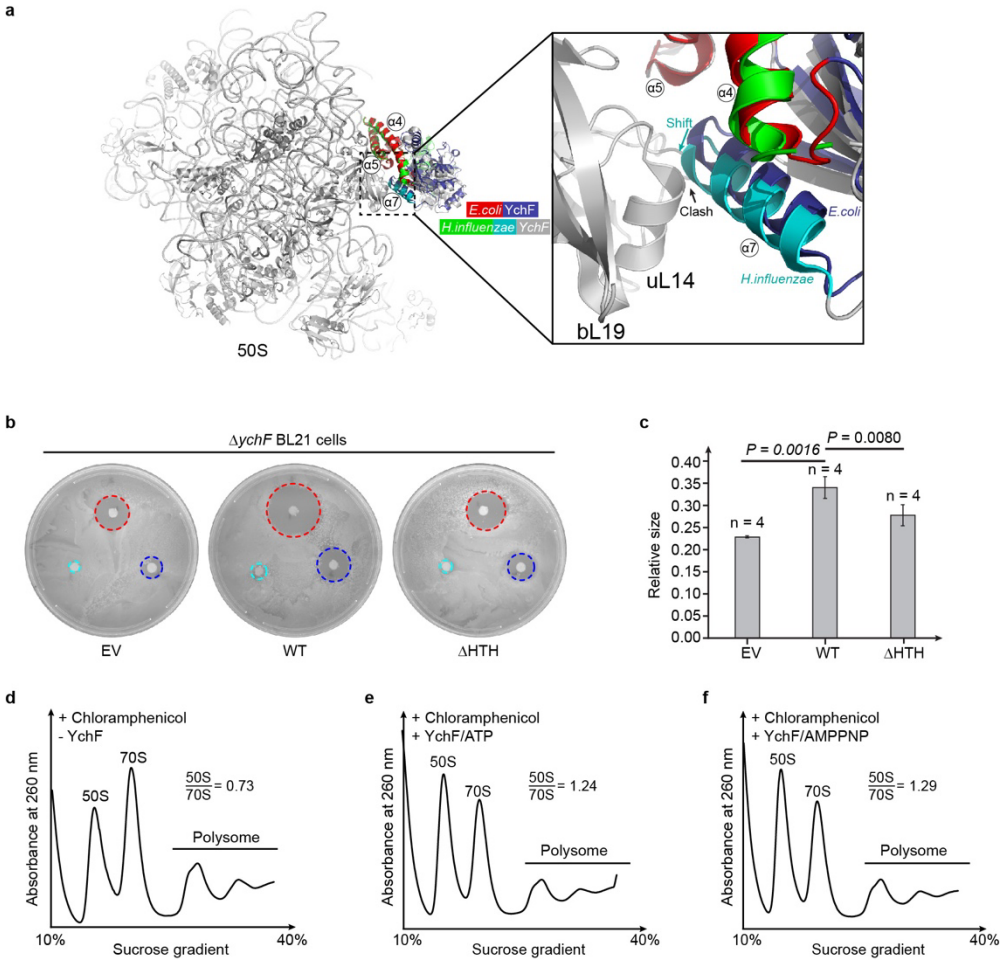
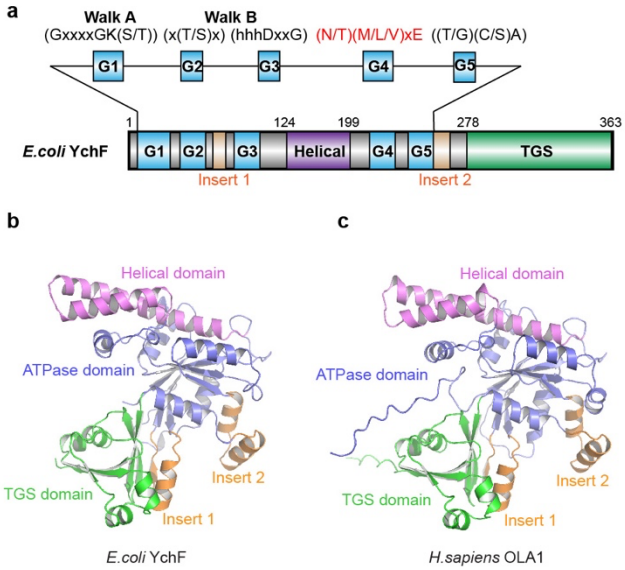


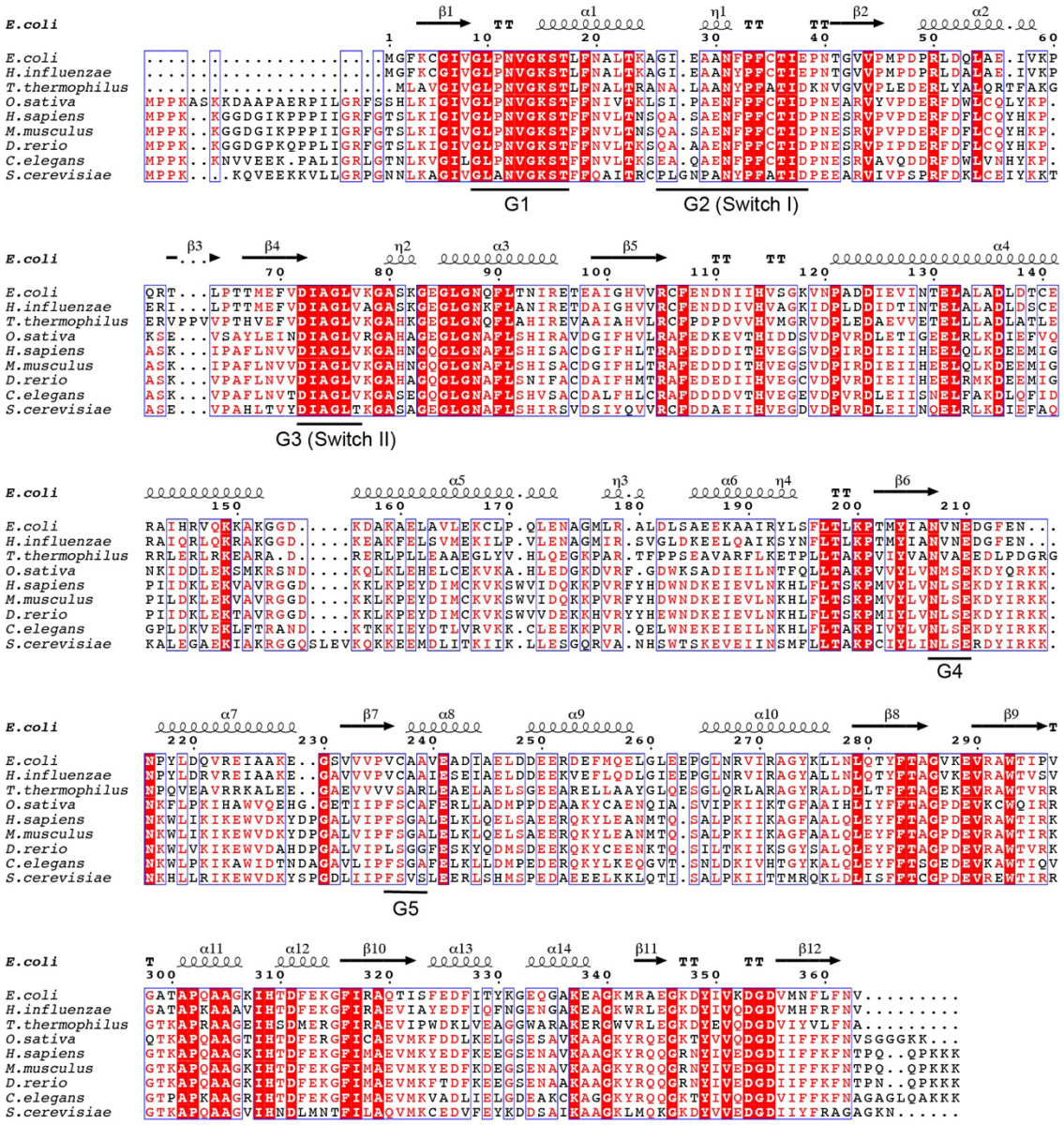
Fig. 3 | YchF promotes the dissociation of ribosomal subunits and the helical domain is required for its cellular function. a, Alignment of helical domain (red) between YchF(H114A) obtained in this study and the crystal structure of *H.influenzae* YchF (PDB: 1JAL). Domains in the two proteins are color-coded and labelled as indicated. **b**, Zones of *E. coli* growth inhibition by disc diffusion away from H_2O_2. 10 mM (cyan), 200 mM (blue) an 800 mM (red) of H_2O_2 were used for the plates expressing wild-type YchF (middle) and the one truncating helical domain (right). Empty vector was used as control (left). **c**, Relative sizes of the red circles in (**b**) are quantified as the ratio of (diameter of red circle)/(diameter of plate). Experiments were performed in replicates (n=4). *P* values were calculated by t-Test with paired two sample for means. EV, empty vector; WT, wild-type; ΔHTH, YchF with α4-α5 coiled coil truncated. **d-f**, Polysome profiling of Δ*ychF* cells with the treatment of chloramphenicol in the absence (**d**) or precence of 5 μM YchF (**e, f**). 2 mM of ATP (**e**) and AMPNP (**f**) were added to the lysis buffer. Ratios of 50S and 70S peaks were calculated based on the peak hights.

11.It is unclear how similar/distant the bacterial and eukaryotic YchF/Ola1 homologs are. I suggest adding a figure/panel showing sequence alignment, and structure alignment if the eukaryotic structure is available.

Response: Thank you for your suggestion. Sequence alignment has been shown in Ext. Fig. 2 and the structure comparison has now been added to Ext. Fig. 1b and c.



Extended Data Fig. 1 | Overview of the domain organization of YchF. a, Domains of *E.coli* YchF are shown in boxes. **b**, **c**, the alphaFold structures of *E.coli* YchF (AF-P0ABU2-F1) and *H.sapiens* OLA1 (AF-Q9NTK5-F1) are shown in cartoon. Domains are color-coded and labeled as indicated.



Extended Data Fig. 2 | Sequence alignment of the universally conserved OLA1/YchF. Multiple sequence alignment of OLA1/YchF from different species using multAlign (http://multalin.toulouse.inra.fr/multalin/).

12.To better explain the rationale for RNA-seq and Ribo-seq, I suggest that the sentence “Next, cells with 80% confluency… and sent for Ribo-seq analysis” is changed to “… and sent for RNA-seq and Ribo-seq analyses to characterize the changes in mRNA abundance and translation”.

Response: Thank you for your suggestion. We have revised the sentence to:

"Next, cells with 80% confluency were incubated with cycloheximide, which is a widely used translation inhibitor to arrest ribosomes on mRNA, and sent for RNA-seq and Ribo-seq analyses to characterize the changes in mRNA abundance and translation efficiency

This clarifies the rationale for using both RNA-seq and Ribo-seq, aligning with your recommendation for improved clarity.

13.In this and other sentences where applicable (e.g. " Results showed that in Δola1 cells, 375 genes were up regulated…"), be more specific regarding methods or results. For example, this sentence could be "RNA-seq data showed that..,". Also, by "vessel development", do you mean "blood vessel development"?

Response: Thank you for the suggestion. We have revised the sentence for clarity. It now reads: "RNA-seq data showed that in *OLA1*$^{-/-}$ cells, 375 genes were up regulated…". Other similar sentences throughout the manuscript have been revised accordingly for clarity. Additionally, the term "vessel development" has been clarified as "blood vessel development" according to the Ext. Fig. 13.

14.Replace "coil-coil" with "coiled coil" or with "alpha4-alpha5 coiled coil" for clarity (using the Greek "alpha").

Response: Thank you for your suggestion. We have revised the manuscript accordingly and replaced the two "coil-coil" with "α4-α5 coiled coil" and "coiled coil" based on its content for clarity.

15. In Table S1, report the structure's correlation coefficient against the maps (CC; global, also report local CC for YchF). In addition, report the statistics for the RNA model (backbone outliers etc). In the Ramachandran plot, are "Outliers" in the allowed or disallowed region?

Response: Thank you for your suggestions. We previously mistakenly referred to CC as FSC. In the revised Table S1, we have corrected this error. Thus, both global and local CC have been reported in the revised Table S1.

Additionally, we have provided statistics for the RNA model, including backbone outliers and other relevant metrics.

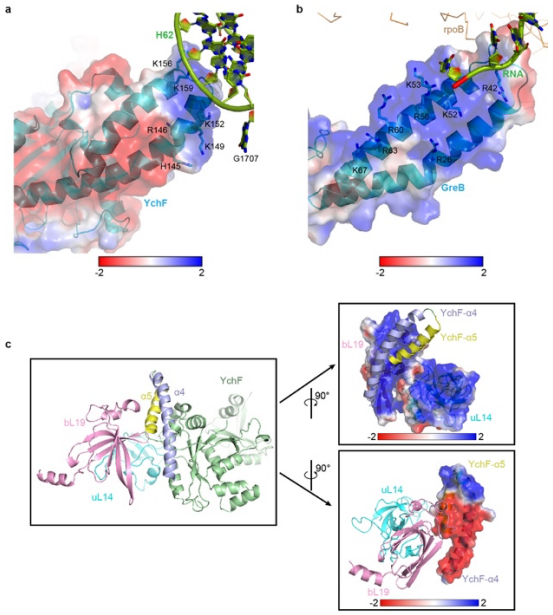
We have rechecked the Phenix results for the Ramachandran plot. The "Outliers" are indeed categorized as "Allowed," and the actual "Outlier" value is 0.00 for both global and local data.

The related part of Table S1 is shown below:

Refinement		
Resolution (Å)	2.4	2.6
Map sharpening B-factor (Å^2)	-20	-20
Correlation coefficient against the maps	0.87	0.86
Rms deviations		
Bond lengths (Å)	0.013	0.010
Bond angles (°)	0.927	0.674
Validation (proteins)		
Molprobity score	1.92	1.62
Clashscore, all atoms	11.40	9.90
Rotamer outliers (%)	0.60	0.20
Validation (RNA)		
Correct sugar puckers (%)	99.5	100
Good backbone conformations (%)	82.28	91.36
Ramachandran plot		
Favored (%)	94.96	97.47
Allowed (%)	4.99	2.53
Outlier (%)	0.00	0.00
Peptide plane		
Cis proline/general (%)	0.0/0.0	0.0/0.0
Twisted proline/general (%)	0.0/0.0	0.0/0.0

16. In Ext. Fig. 6, what are the units shown on the electrostatic potential heatmap? These numbers (around 80) look strange, as they are unlikely to correspond to the local charge units (which are usually on the order of 1-2 electrons/protons).

Response: Thank you for your comments. We previously used PyMOL for calculations, and in the revised Ext. Fig 6, we utilized the PyMOL APBS plugin for these computations, setting the range to -2 to 2 (as shown below). The related description has been added to the figure legend in the revised manuscript.



Extended Data Fig.6

17.Proofread the manuscript to correct numerous typos and stylistic errors – including Methods (e.g. “Holley” and many more typos).

Response: Thank you for your feedback. We have proofread the manuscript to correct typos and stylistic errors, including in the Methods section. The correction of the English language has also been completed by my native English-speaking colleague.

Reviewer #3 (Remarks to the Author):

This paper uses a suite of experimental approaches to uncover the molecular workings of Ola1/YchF Obg-Like GTPases from E. coli, yeast (unclear the species; I assume it is S. cerevisiae) and H. sapiens. These factors are well-known to be involved in resistance to stress, but the biological function is less clear, although both bacterial and eukaryotic factor were earlier implicated in translation, and direct interaction with the ribosome was shown. The authors use a melange of approaches: cryoEM (in E. coli), RNA-Seq and Ribo-Seq (in HeLa), phenotypic assays (different in different experimental systems) and molecular biology (ribosome splitting assays using yeast lysates). The result of this effort is rather confusing, with the individual parts being somewhat disconnected from each other. The paper reads like three papers assembled sequentially and this collection would be well-suited for a special issue on this protein family in a specialised journal. I have several specific suggestions:

Response: Thank you for the comments. We have revised the description of our results, removed the yeast data and enhanced the connection between the *E. coli* and HeLa cell results. Additionally, we validated our findings using the mCherry-D12-GFP reporter system in both *E. coli* and HeLa cells, obtaining consistent results. The revised manuscript now presents a clearer logical flow and stronger evidence for the role of YchF/OLA1 in promoting the translation of D/E-rich mRNA.

-The three proteins studied in the three experimental systems are all from the Obg-Like protein family. However, it is unclear if these proteins have the same exact function in eukaryotes and bacteria. Therefore, the key results from different systems should be cross-validated between the experimental systems and followed up with orthogonal methods. Stalling on D/E-rich regions observed in HeLa lacking Ola1 should be i) validated in yeast and bacteria ii) tested using stalling reporters.

Response: Thank you for your comments. We have addressed these concerns by further validating our findings across systems. Specifically, we have now conducted additional experiments to cross-validate the results in both human and bacteria. Stalling on D/E-rich regions has been tested using stalling reporters in *E. coli* and HeLa systems, confirming consistent stalling effects. These additional validations, strengthen our conclusions about the conserved role of Obg-like proteins across different organisms. Please see the response for comment #2 for details.

-Currently the yeast data does not add much. Validating the structural data obtained with E. coli in E. coli system would make much more sense. Polysome profiles, genetic interactions with other ribosome rescue systems, stalling reporters are the assays one would use.

Response: Thank you for the suggestion. We agree that validating the structural data obtained in *E. coli* within the *E. coli* system itself would be more meaningful and provide stronger insights. To focus on *E.coli* and Hela cells, we have removed the yeast data (which only contains the spot assay and does not add much to the conclusion) in the revised manuscript.

To further verify the function of OLA1/YchF on D/E-rich mRNA, stalling reporter (mCherry-D12-GFP) system was used (the revised Fig.6). Alignment of the TGS domain between YchF, Rbg1 and RelA was also included in the revised manuscript. Rbg1 was reported to promote the translation on R/K-rich mRNA (reference #1) and RelA is involved in stringent response activation (reference #2). Both could interact with h5 of the small subunit rRNA on the ribosome (the new Ext. Fig. 15). This suggests that YchF may share similarities with other ribosome rescue systems. Additionally, deletion of the TGS domain omitted the splitting activity of YchF (the new Ext. Fig. 16), further showed that the binding of YchF to stalled ribosome with TGS domain is possible.

Reference #1: Zeng, F. et al. Conserved heterodimeric GTPase Rbg1/Tma46 promotes efficient translation in eukaryotic cells. *Cell Rep* 37, 109877 (2021).

Reference #2: Loveland, A. B. et al. Ribosome•RelA structures reveal the mechanism of stringent response activation. *Elife* 5 (2016).

The revised results and discussion about the reporter assay and TGS domain are listed below:

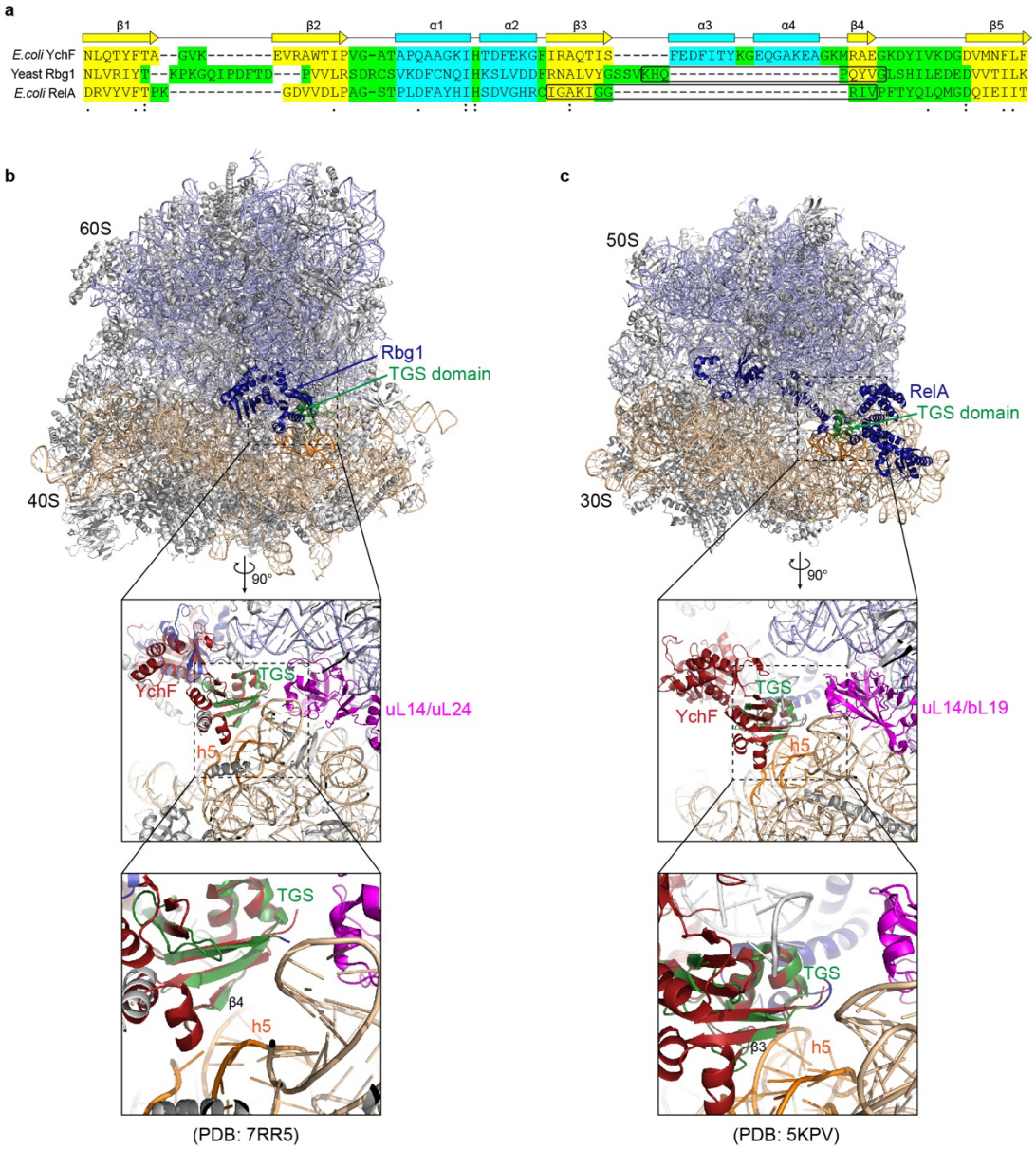
"To verify the importance of OLA1/YchF on the translation of D/E-rich mRNA, we constructed mCherry-D_{12}-GFP reporter system and measure the value of GFP/mCherry in WT or Δ*ychF*/*OLA1*$^{-/-}$ cells (Fig. 6a-c). Results showed that deletion of OLA1/YchF causes an increase in GFP/mCherry level, which can be interpreted as the ribosome-associated quality control (RQC) pathway being activated, leading to the degradation of mCherry protein.

The reported complex in our study represents likely the post splitting complex. How does OLA1/YchF recognize the stalled ribosome and promote the splitting? We noticed that the TGS domains of Rbg1 and RelA both could interact with h5 of the 18S/16S rRNA on the 80S/70S

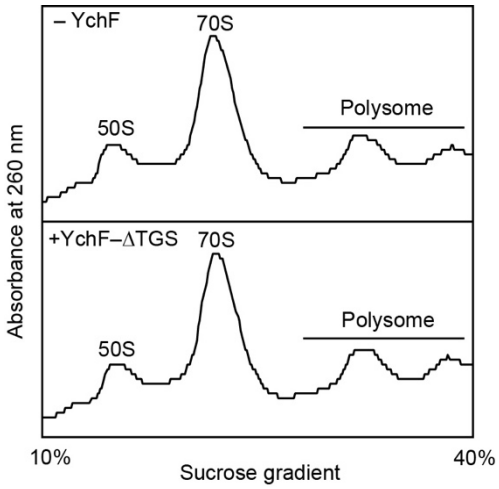
ribosome[25,42] (Extended Data Fig. 15). Structure based alignment of TGS domain in YchF, Rbg1 and RelA showed that their sequences are not conserved but structures are similar (Extended Data Fig. 15a). Rbg1 uses the loop after β3 and partial β4 to interact with h5 (Extended Data Fig. 15b). RelA adopts similar pattern but including β3 for the interaction (Extended Data Fig. 15c). Superimposing of the TGS domain on the ribosome showed that YchF may also be able to bind to this region. Since h5 is closed to the uL14/bL19, which is the binding site of YchF in 50S subunit. We hypothesized that OLA1/YchF might initially bind to the stalled ribosome via its TGS domain, possibly in conjunction with other ribosome-associated factors like Pth. Subsequently, through certain changes, the large and small subunits may be split, with OLA1/YchF remaining on the large subunit to prevent re-association with the small subunit. This process resembles how, after the RQC trigger complex (RQT) separates the 60S from 40S, eIF6 binds to the 60S, preventing the formation of 80S[26].”

“Alignment of the TGS domain between YchF, Rbg1 and RelA suggested a possible binding site of OLA1/YchF on the stalled ribosome, which is h5 of the small subunit rRNA. We still do not know the details of the splitting process by lacking the structures of the pre-splitting complexes. However, based on the fact that YchF(H114A) binds to the 50S subunit, ATPase activity is most likely not required for ribosomal subunit dissociation, but important for YchF dissociation from the 50S subunit.”

The revised Fig.6 for reporter assay and the new Ext. Fig. 15 and 16 for TGS alignment are listed below:



Extended Data Fig. 15 | YchF might target to 70S by TGS domain. a, Sequence alignment of the TGS domain of *E.coli* YchF, *S.cerevisiae* Rbg1 and *E.coli* RelA. The sequence alignment was based on the results of the structural alignment. Helix, α-strand and loop are colored cyan, yellow and green, respectively. The binding sites of TGS to the h5 of 16S rRNA are marked with black boxes. The symbol '.' and ':' indicates the similar and identical amino acids, respectively. **b**, **c**, Superimposing YchF on Rbg1 (**b**) and RelA (**c**) based on the TGS domains. Rbg1 and RelA are shown in blue with TGS domain in green, while YchF is depicted in red. The binding site of YchF on 50S in this study are colored magenta. For clarity, the hydrolase (HYD) and synthetase (SYN) domains of RelA have been hidden in the enlarged subfigure (**c**).



Extended Data Fig. 16 | Deletion of TGS domain omits the splitting activity of YchF. Polysome profiling of Δ*ychF* cells with the treatment of chloramphenicol in the absence (upper) or precence of 5 μM YchF-ΔTGS with 2 mM of ATP.

-The connections between the mechanism and the phenotypes are unclear and confuse the reader further.

Response: To clarify the links between mechanism and phenotypes, we have improved the explanation in the revised manuscript, ensuring a clearer narrative that aligns the mechanistic details with the observed phenotypes. This helped prevent confusion and provide a stronger, more coherent connection between the experimental findings and the underlying biological processes. Thank you for pointing this out.

-The word 'regulate' is misused pervasively. The function of the factor seems to be resolving ribosomal stalling. Loss of the factor through genetic disruption is not 'regulation' as the authors do not demonstrate the existence of a specific response that regulates the expression level / activity of the factor in response to ribosomal stalling.

Response: Thank you for this insightful comment. We agree with your observation. In response, we have revised the manuscript to clarify that the factor's role is primarily in **resolving ribosomal stalling** rather than regulating it. To ensure more accurate expression, we have changed the title to:

"Conserved ATPase OLA1 promotes efficient translation on D/E-rich mRNA".

-Introducing the relevant literature on ribosomal stalling and the multiple factors that rescue stalled ribosomes is essential. In general the Introduction is exceedingly short.

Response: Thank you for your comment. We have expanded the Introduction section to include a comprehensive overview of the relevant literatures on ribosomal stalling and the various factors involved in rescuing stalled ribosomes. The revised part is:

"Here, we reported that OLA1/YchF binds to the ribosomal large subunit and promotes the efficient translation on D/E-rich mRNA. Disruption in the translation can be caused by the truncated mRNA or mRNA with a specific structure/sequence[23]. Rescuing the stalled ribosome on these mRNAs need numerous ribosome-associated proteins to release the nascent peptide and split the ribosome. For instance, the proteins Dom34, Hbs1 and Rli1 target ribosomes with truncated mRNA in eukaryotes, catalyzing the splitting of the subunit [23]. While stalls on specific mRNA structure/sequence can be resolved under optimal conditions, i.e., synthesis of proline-rich is made possible by EFP (in bacteria, eIF5A in eukaryotes and archaea)[24]. Stalling on R/K-rich mRNA is promoted by Rbg1/Tma46 complex or target/split by RQT complex if ribosome collision occurs[25,26]. Currently, there is limited research on D/E-rich mRNA. Through structural analysis and Ribo-seq data, we speculated that OLA1/YchF can enhance translation on D/E-rich mRNA, a notion validated by our constructed reporter. OLA1/YchF binds to the large subunit at the uL14/bL19 position via its helical domain, preventing the re-association of split ribosomes. These findings not only provide deeper insights into OLA1/YchF function but also contribute in better understanding the regulation of translation on D/E-rich mRNA."

-Nomenclature: ΔychF in bacteria but ola1Δ in yeast (note the positioning of Δ). In human cell lines no Δ is used; homozygous KO should be referred to as superscripted –/–, and the gene name should be capitalised.

Response: Thank you for your comment. We have updated the nomenclature in the revised manuscript.

Blue: Responses

Cyan: Revised texts from last time

Red: Revised texts for this time

Point-to-point Responses to the Reviewer #1:

The manuscript "Conserved GTPase OLA1 promotes efficient translation on D/E-rich mRNA" reports the structure of the highly conserved GTPase Ola1/YchF, which is a member of the TRAFAC (translation factors) class of GTPases. The molecular mechanism and cellular function of Ola1 and its bacterial homolog YchF have been elusive for decades but mounting biochemical evidence indicates a ribosome associated role. Recent biochemical studies using UV inducible crosslinkers locate Ola1/ YchF to the E-site on the 30S small ribosomal subunit. The work reported in this manuscript uses affinity purification of a his-tagged catalytically inactive variant to enrich for YchF-Ribosome complexes. Initial biochemical studies by the authors indicate low complex occupancy which they seek to overcome by the use of the inactive variant for enrichment and subsequent glutaraldehyde crosslinking to stabilize the enriched YchF-50S ribosomal complex. The resulting structure provides the first direct visualisation of YchF on the ribosome and locates it close to the A-site in the 50S ribosomal subunit.

Response: Thanks for your thoughtful comment. OLA1/YchF is indeed a multifunctional protein with diverse roles. As you noted, previous studies using UV crosslinking have reported its binding to the E-site on the 30S subunit. In addtion to this, studies by Becker, M. (2012) have shown that the 70S ribosome can enhance YchF's ATPase activity. Furthermore, YchF/Ola1 has also been found to interact with factors such as eIF2 (Chen, H., 2015) and Hsp70 (Mao, R.F., 2013). These varied functions are likely context-dependent, influence by the specific cellular environment. In our experimental conditions (YchF was overexpressed and cells were grown at 16 ˚C), YchF appears to predominantly associate with the 50S subunit. We have added a detailed discussion on this point to the revised *Introduction* and *Discussion* section to clarify our interpretation and align our findings with previous studies. Thank you for highlighting this important aspect.

Although crosslinking was used to stabilize the complex, we believe that the binding of YchF to the 50S subunit is specific. This conclusion is supported by the two-step purification process, including pulldown and sucrose cushion centrifugation, performed prior to the addition of the crosslinker, which effectively eliminates nonspecific binding of YchF. Furthermore, the results of the anti-association/splitting experiments are consistent with the functional prediction based on the structure of YchF bound to the 50S subunit.

Becker, M., Gzyl, K.E., Altamirano, A.M., Vuong, A., Urban, K. and Wieden, H.J. (2012) The 70S ribosome modulates the ATPase activity of Escherichia coli YchF. RNA Biol, 9, 1288-1301.

Chen, H., Song, R., Wang, G., Ding, Z., Yang, C., Zhang, J., Zeng, Z., Rubio, V., Wang, L., Zu, N. et al. (2015) OLA1 regulates protein synthesis and integrated stress response by inhibiting eIF2 ternary complex formation. Sci Rep, 5, 13241.

Mao, R.F., Rubio, V., Chen, H., Bai, L., Mansour, O.C. and Shi, Z.Z. (2013) OLA1 protects cells in heat shock by stabilizing HSP70. Cell Death Dis, 4, e491.

Revised Introduction:

OLA1/YchF is involved in different cellular stress response pathways, such as heat shock, integrated stress, cell adhesion, and antioxidant responses[8,9]. It is also implicated in the regulation of tumor progression in numerous types of cancer including breast, lung, and hepatocellular cancer[10-12]. Accumulating reports have shown that OLA1 is highly expressed in most cancers and could be a potential biomarker and therapeutic target for cancer[10,13-15]. OLA1 also works as an intrinsic stress response regulator, including oxidative stress[6] and heat shock[16,17]. Heat shock protein 70 (HSP70) is a key molecule in different types of cancer, and high expression of HSP70 is associated with poor tumor progression[18]. OLA1 regulates HSP70 protein stability to inhibit shock-induced cell death[16]. OLA1 also interacts with BRCA1 and BRCA1-associated RING domain protein 1, wihch affects centrosome function and is suspected to lead to carcinogenesis in hereditary breast and ovarian cancer[11,19,20].

Growing evidence showed that OLA1/YchF plays important functions in translation regulation on a subset of mRNAs, such as leaderless mRNAs encoding stress response proteins for cell survival[21]. Human OLA1 has been implicated in translation initiation by interacting with eIF2, thereby preventing its binding to the initiator methionyl-tRNA[22]. Conversely, under stress conditions, the down-regulation of OLA1 has been shown to enhance translation initiation and mitigate the integrated stress response (ISR)[22,23]. A recent study in *Mollicutes* also found that YchF is one of the 104 core set proteins that can sustain ribosome biogenesis and translation of the genetic code in self-replicating bacteria with reduced genomes[24]. Ribosome binding of OLA1/YchF has been observed in *E.coli*[21,25-27], yeast[17], Trypanosoma[4], plants[7], and human cells[22]. The available data suggest that YchF/OLA1 binds not only to fully assembled ribosomes but also independently to the small and large ribosomal subunits. And the 70S ribosome was reported to be able to stimulate the ATPase activity of YchF[26]. Recent biochemical studies using UV inducible crosslinkers locate YchF to the E-site on the 30S small ribosomal subunit[28]. Despite these advances, questions remain unanswered regarding the molecular details of ribosome binding and translation regulation, as well as the exact roles of OLA1/YchF during these processes. One major reason can be attributed to the lack of structures that catch the OLA1/YchF in the complex with ribosomes.

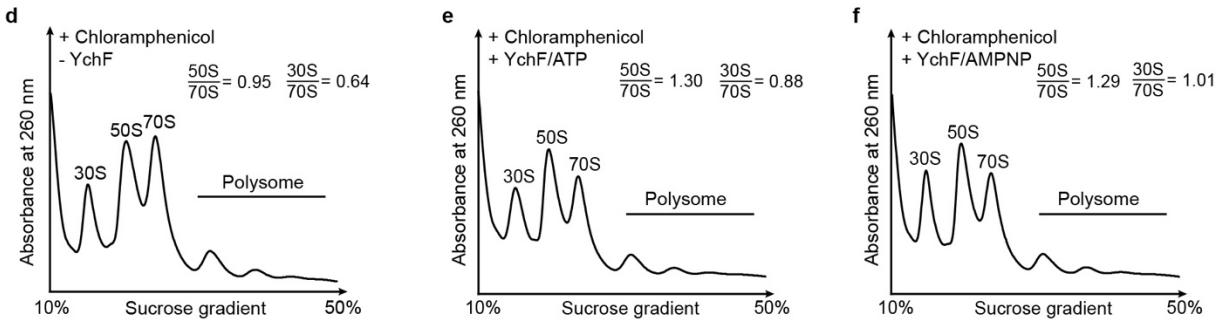
Revised Discussion:

Through the anti-association and splitting assays, we proposed that OLA1/YchF may facilitate the dissociation of stalled ribosomes at D/E-rich regions through specific mechanisms, thereby ensuring efficient translation of D/E-rich mRNA. Since many ribosomal proteins contain D/E-rich sequences, the absence of OLA1/YchF could lead to the formation of heterogeneous ribosomes, ultimately resulting in aberrant expression of genes involved in processes such as membrane formation, chromatin organization, and hypoxia response. However, it is important to remember that OLA1/YchF is a multifunctional protein. In addition to binding the large ribosomal subunit, it can regulate translation through other mechanisms[22,28,61,62] and influence cell growth by interacting with proteins like HSP70[2,16,63,64]. Therefore, when studying the effects of YchF/OLA1 on cellular function, a comprehensive approach considering multiple perspectives is essential.

Based in the locations the authors hypothesise a potential function in ribosome splitting and/or reassociation prevention. The authors attempt to support this by a reconstituted splitting experiment followed by density gradient centrifugation, in which they see an increased ration of 50S to 70S, which the authors claim suggests splitting (disassembly of the 70S ribosomes into the constituting the 30S and 50S subunits). However, the experiment fails to report 30S subunits (which is easily obtainable using density gradient centrifugation) and the authors therefore do not correlate the changed 50S:70S ratio with an expected proportional increase in the 30S:70S ratio, maintaining a 1 to1 30S:50S ratio. Since no additional biochemical (e.g. kinetic observation of splitting or inhibition of re-association of the subunits) is provided to supplement this, the data does not support the claim of a splitting or anti-association activity of YchF.

Response: Thanks for your insightful comment. We have repeated the sucrose gradient centrifugation experiment and obtained improved profiles, where both the 30S and 50S peaks are now clearly observed (as shown in the revised Figure 3d-f). Compared to the control, the addition of YchF resulted in an increase in the peaks for both the 30S and 50S subunits by approximately 1.4- to 1.6-fold. These updated results better demonstrate the correlation between the changes in the 50S:70S ratio and the corresponding increase in the 30S:70S ratio, supporting the proposed role of YchF in ribosome splitting or anti-association.

Revised Figure 3d-f.



Revised Results:

Considering that the YchF/50S complex was purified by pulldown, we turned to investigate the effects of YchF on ribosomes in cells. As shown in Fig. 3d, additional of chloramphenicol to the Δ*ychF* cells could stabilize the polysome peaks in the sucrose gradient[45]. When YchF was added to the cell extracts, increased subunit peaks were observed in the presence of both ATP and AMPNP (Fig. 3e, f). In the absence of YchF, the ratio of 30S and 50S to 70S were measured as 0.64 and 0.95, respectively. However, in the presence of YchF under ATP condition, these ratios increased to 0.88 and 1.30, and under AMPPNP conditions, they increased to 1.01 and 1.29 (Fig. 3d-f). Both the 30S and 50S peaks showed an approximate 1.4- to 1.6-fold increase, suggesting that these increases are derived from the splitting of 70S ribosomes.

To investigate the function of Ola1/YchF the authors then switch from the bacterial system to Eukaryotes and perform mRNA-seq in HeLa cells. The respective analyses identify several differentially regulated genes which the authors claim seems to be consistent with previous reported effects of Ola1/YchF. Using Ribo-seq the authors then identified a role of OLA1/YchF in the translation of D/E-rich mRNA.

The flow of the MS has significantly improved but this reviewer is still confused about the importance for the section “Deletion of OLA1 increases the migration rate of Hela cells”

Response: Thanks for your feedback. You are correct that in this section, we briefly described the effect of OLA1 deletion on the migration rate of Hela cells. The primary purpose of this section is to establish a connection between the observed cellular phenotype and the subsequent RNA-seq and Ribo-seq results, which demonstrate that OLA1 deletion impacts the translation of genes

across multiple pathways. We apologize for any confusion caused. To clarify the focus of this section, we have revised its title in the manuscript to: *"*Deletion of OLA1 Alters the Translation of Genes Across Diverse Pathways.*"* We hope this change better reflects the main emphasis of the section.

Blue: Responses

Cyan: Revised texts from last time

Red: Revised texts for this time

Point-to-point Responses to the Reviewer #2:

The authors have most of my concerns, and the revised manuscript reads better than the initial submission. Several issues, however, remain to be addressed:

1. The authors claim that the buried surface area of 643 A^2 represents "a large interface among protein complexes". They refer to a study presenting a machine-learning-based model for predicting binding affinities from buried surface areas. It is unclear how this work is relevant; perhaps the authors used this model to predict the affinity of YchF – which they should clarify. Nevertheless, if compared with other protein complexes, the BSA of ~650 A^2 would be on the lower end of binding affinities (as complexes generally have the BSAs of 500 to 2000, and higher than 2000 for very stable complexes)

- https://onlinelibrary.wiley.com/doi/10.1002/pro.2230

Response: Thanks for your comment. We have revised the sentence as follows:

"By analyzing the interface using the 'Protein interfaces, surfaces and assemblies' (PISA) service at the European Bioinformatics Institute[29], the area of these interfaces was calculated to be more than 643 Å^2. This accounts for a reasonable interface among protein complexes[30,31], suggesting a stable binding of YchF to the 50S subunit."

To address your concern, we carefully compared the following two publications:

Ref1 (cited in our manuscript): Yang, Y.X., Wang, P. and Zhu, B.T. (2022) Importance of interface and surface areas in protein-protein binding affinity prediction: A machine learning analysis based on linear regression and artificial neural network. Biophysical Chemistry, 283, 106762.

Ref2 (referred by reviewer, also cited in the revised manuscript): Chen, J., Sawyer, N. and Regan, L. (2013) Protein-protein interactions: general trends in the relationship between binding affinity and interfacial buried surface area. Protein Sci, 22, 510-515.

Figures relevant to our analysis have been adapted from these two studies:[REDACTED]

(Adapted from Ref1 Figure 3C) [REDACTED]

(Adapted from Ref2 Figure 1A) [REDACTED]

Here are the conclusions:

(1). Comparison of Figures from Ref1 and Ref2:

Althrough Ref1 utilized a comprehensive model in its final analysis, Figure 3C in Ref1 specifically presented the original data with linear fitting, which is comparable to Figure 1A in Ref2. Thus, both figures can be reasonably compared.

(2). Differences in Interface/Buried Area values between the two figures:

The interface areas reported in Ref1 range from 300-1400 $Å^2$, while those in Ref2 range from 300-3400 $Å^2$. Notable, for a Kd of 1 μM, the interface area if Ref1 was approximately 600 $Å^2$, whereas in Ref2 it is around 1200 $Å^2$. This discrepancy arises because the two studies employed different software to calculate interface areas: Ref1 used "$Q_{contact}$", while Ref2 used "NACCESS v2.1.1". In contrast, our study used "PISA" for these calculations.

For example, for PDB:2WWX, Ref2 reported a buried area of 3393 $Å^2$ using NACCESS, while PISA calculated the interface area as 1693 $Å^2$.

For PDB:1AY7 (Kd = 1 μM) from PDBbind, PISA reported an interface area of 616 $Å^2$, which aligns closely with the data in Ref1 Figure 3C. For PDB: 1EFN (Kd = 380 nM) from PDBbind, PISA reported 629 $Å^2$, which is nearly identical to $Q_{contact}$'s reported value of 616 $Å^2$.

These observations indicates that PISA and $Q_{contact}$ yield similar interface area values, whereas NACCESS reports values approximately 2-fold higher, which explains the differences between the figures in Ref1 and Ref2.

(3). Rationale for Using Ref1:

Since PISA results are more consistent with those from $Q_{contact}$, we have chosen to retain Ref1 in our revised manuscript. For the interface between YchF and the 50S subunit, PISA reported an interface area of 643 $Å^2$. When fitted to the linear model in Ref1, this corresponds to a Kd of approximately 175 nM.

Most binding interface areas in Ref1 are in the range of 400-800 $Å^2$ (compared to 800-1600 $Å^2$ in Ref2). Therefore, we conclude that the interface between YchF and 50S subunit is "reasonable", and the binding is "stable".

2. In the section describing the helical domain of YchF, the authors state that "YchF is a multifunctional protein". The molecular functions of YchF, as described in the introduction, are primarily linked to translation and depend on ribosome binding. In this work, the authors propose that they have elucidated the molecular mechanisms of YchF function, which is to to bind to the large subunit to promote ribosome dissociation. What other functions of "multifunctional YchF" do the authors refer to in this section? They need to elaborate on that and explain the importance of the 50S binding function relative to other functions.

Response: Thanks for your comment. As noted, YchF is indeed a multifunctional protein, with its molecular functions extending beyond ribosome-related activities. We have added this background into the revised introduction. In this work, we focus on YchF's ribosome-associated function, specifically its binding to the 50S subunit and its proposed role in promoting ribosome dissociation. We have added a discussion in the revised manuscript to elaborate on these additional functions of YchF and to contextualize the importance of its 50S binding function relative to its other roles.

We appreciate your feedback, which has helped us clarify this important aspect of YchF's multifunctionality.

Revised introduction:

OLA1/YchF is involved in different cellular stress response pathways, such as heat shock, integrated stress, cell adhesion, and antioxidant responses[8,9]. It is also implicated in the regulation of tumor progression in numerous types of cancer including breast, lung, and hepatocellular cancer[10-12]. Accumulating reports have shown that OLA1 is highly expressed in most cancers and could be a potential biomarker and therapeutic target for cancer[10,13-15]. OLA1 also works as an intrinsic stress response regulator, including oxidative stress[6] and heat shock[16,17]. Heat shock protein 70 (HSP70) is a key molecule in different types of cancer, and high expression of HSP70 is associated with poor tumor progression[18]. OLA1 regulates HSP70 protein stability to inhibit shock-induced cell death[16]. OLA1 also interacts with BRCA1 and BRCA1-associated RING domain protein 1, wihch affects centrosome function and is suspected to lead to carcinogenesis in hereditary breast and ovarian cancer[11,19,20].

Growing evidence showed that OLA1/YchF plays important functions in translation regulation on a subset of mRNAs, such as leaderless mRNAs encoding stress response proteins for cell survival[21]. Human OLA1 has been implicated in translation initiation by interacting with eIF2, thereby preventing its binding to the initiator methionyl-tRNA[22]. Conversely, under stress conditions, the downregulation of OLA1 has been shown to enhance translation initiation and mitigate the integrated stress response (ISR)[22,23]. A recent study in *Mollicutes* also found that YchF is one of the 104 core set proteins that can sustain ribosome biogenesis and translation of the genetic code in self-replicating bacteria with reduced genomes[24]. Ribosome binding of OLA1/YchF has been observed in *E.coli*[21,25-27], yeast[17], Trypanosoma[4], plants[7], and human cells[22]. The available data suggest that YchF/OLA1 binds not only to fully assembled ribosomes but also independently to the small and large ribosomal subunits. And the 70S ribosome was reported to be able to stimulate the ATPase activity of YchF[26]. Recent biochemical studies using UV inducible crosslinkers locate YchF to the E-site on the 30S small ribosomal subunit[28]. Despite these advances, questions remain unanswered regarding the molecular details of ribosome binding and translation regulation, as well as the exact roles of OLA1/YchF during these processes. One major reason can be attributed to the lack of structures that catch the OLA1/YchF in the complex with ribosomes.

Revised Disccusion:

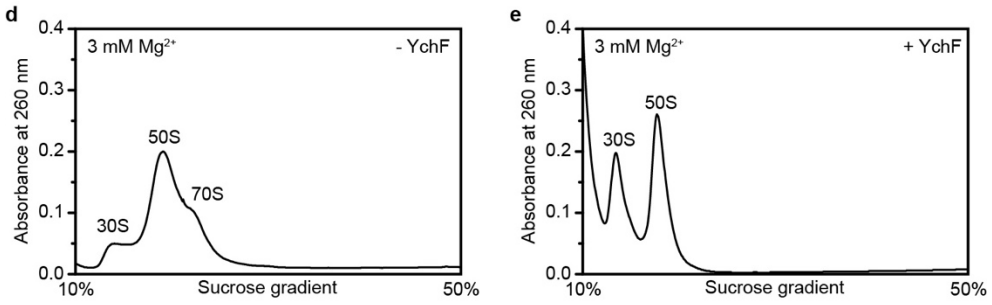
Through the anti-association and splitting assays, we proposed that OLA1/YchF may facilitate the dissociation of stalled ribosomes at D/E-rich regions through specific mechanisms, thereby ensuring efficient translation of D/E-rich mRNA. Since many ribosomal proteins contain D/E-rich sequences, the absence of OLA1/YchF could lead to the formation of heterogeneous ribosomes, ultimately resulting in aberrant expression of genes involved in processes such as membrane formation, chromatin organization, and hypoxia response. However, it is important to remember that OLA1/YchF is a multifunctional protein. In addition to binding the large ribosomal subunit,

it can regulate translation through other mechanisms[22,28,61,62] and influence cell growth by interacting with proteins like HSP70[2,16,63,64]. Therefore, when studying the effects of YchF/OLA1 on cellular function, a comprehensive approach considering multiple perspectives is essential.

3. In Supp. Fig. 9, the authors show that YchF fails to split the 70S, by incubating the ribosomes with the "5-fold excess" of YchF. Given that the binding affinity is likely low, have they tried a much higher excess (e.g. 100-fold)? This would support their conclusion that YchF is indeed an anti-association factor.

Response: Thanks for your insightful surggestion. In the revised manuscript, we performed the experiments using a 100-fold excess of YchF. The results demonstrated that YchF indeed promotes the splitting of 70S ribosomes under these conditions, further supporting its role as an anti-association factor. We have updated the results and discussion sections accordingly to reflect these findings. Thank you again for your valuable feedback.

Revised Extended Data Fig.9d and e



d, Sucrose gradient profile of the 70S at semi-dissociation conditions (3 mM Mg^{2+}) with the equilibrium shifted toward the association. **e**, Sucrose gradient profile of the 70S at 3 mM Mg^{2+} upon addition of 100-fold excess of YchF.

Revised Methods:

For splitting on purified ribosomes, 0.3 μM 70S were incubated with 100-fold excess of YchF and 3.3 mM ATP on ice for 30 min. Reactions were then layered onto a 10-50% (w/v) sucrose gradient in buffer C (20 mM HEPES-NaOH, pH 7.4, 50 mM NH_4Cl, 3 mM $Mg(OAc)_2$) and further

centrifuged in SW 40Ti rotor with 38,000 rpm, 4 ˚C for 3.5 h. Purified 70S without incubating with YchF was used as control. Peaks of ribosomes were collected and monitored at 260 nm.

For splitting on the stalled ribosomes, the Δ*ychF* cells were first grown to OD_{600} of 0.6 and treated with 200 μg/mL chloramphenicol for 2 min.

Revised Results:

We then set out to examine whether YchF could split ribosomes or prevent the formation of 70S. The incubation of purified YchF with 70S did show an increment of 30S and 50S peaks in the sucrose gradient experiments when 100-fold excess of YchF was used (Extended Data Fig. 9d and e). Lower amount (5-fold excess) of YchF did not increase the splitting of 70S. These results indicated that YchF binds weakly to the purified ribosomes or subunits, which is consistent with the binding assay shown in Extended Data Fig. 3a.

4. The description of the experiment mCherry-D12-GFP experiment is misleading and the data in human cells do not support the conclusion that OLA1 affects the D12 reporter: the is no difference in GFP/mCherry between WT and OLA1-/- cells (Fig. 6a-c).

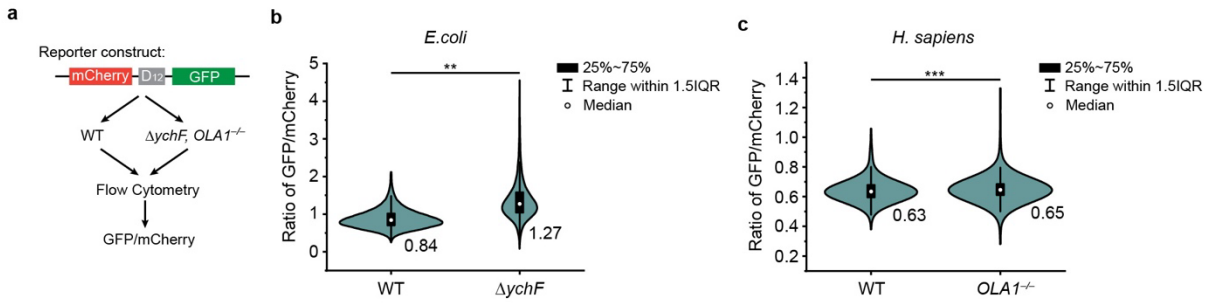
Response: Thanks for your comment. In the revised manuscript, we have included the median values for each plot.

As shown in the figure, the median values for *E.coli* WT and Δ*ychF* are 0.84 and 1.27, respectively. The Wilcoxon test performed using Origin software indicated that the two distributions are significantly different at the 0.05 level.

For Hela cells, the median values for WT and *OLA1*$^{-/-}$ are 0.63 and 0.65, respectively. The Wilcoxon test showed that the two distributions are significantly different at the 0.001 level.

Although the increase in the median value for *OLA1*$^{-/-}$ is modest, the significant difference between the distributions suggests that OLA1 affects the D12 reporter in Hela cells. The small difference may be attributed to the complex regulatory mechanisms in human cells.

Revised Figure 6a-c



5. In Fig. 2, it is unclear which species were used for sequence alignment and WebLogo. It is not clear how the species were selected for multiple sequence alignments in this work. This needs to be clarified in figure legends and Methods.

Response: Thank you for your feedback. We apologize for the lack of clarity regarding the species used for sequence alignment and WebLogo in Figure 2. The species were selected based on the results of UniProt (https://www.uniprot.org/) searching. The 28 reviewed items with the name of 'YchF' or 'OLA1' were used for the alignment. Sequences in the helical domain were chose for the WebLogo. The full list of the species with their sequences were attached as 'Supplementary Info 1' in the revised manuscript.

The revised figure legends:

g, Sequence logos created in WebLogo shows that lysine and arginine are conserved in the tip of the helical domain among species. A total of 28 OLA1/YchF sequences from different species were used for the alignment.

The revised methods:

Multiple sequence alignment

The protein sequences of OLA1/YchF from *E.coli*, *H.influenzae*, *T.thermophilus*, *O.sativa*, *H.sapiens*, *M.musculus*, *D.rerio*, *C.elegans* and *S.cerevisiae* were obtained from Uniprot (https://www.uniprot.org) and the sequence alignment were performed in multAlin

(http://multalin.toulouse.inra.fr/multalin/). The alphaFold structure of *E.coli* YchF (AF-P0ABU2-F1) was used to obtain the information of secondary structure.

To generate the sequence logos, all the 28 reviewed items tagged OLA1 or YchF in Uniprot were downloaded and the sequence of helical domain was extracted based on their multiple sequence alignment (Supplementary Info 1). Sequence logos were then generated in WebLogo (https://weblogo.berkeley.edu/logo.cgi) using the extracted sequences.

6. In Methods, what was the complex concentration in the 3 uL sample aliquots applied to cryo-EM grids?

Response: Thanks for your comment. The concentration we used was 150 nM. We have clarified this information in the revised manuscript to ensure accuracy and reproducibility.

7. The manuscript, including Methods and other sections, needs to be further carefully proofread (e.g. “Laplacin-of-Guassin”, “palys” and more).

Response: Thank you for pointing this out. We apologize for the typographical errors and have carefully proofread the entire manuscript, including the Methods and other sections. Corrections have been made to address errors such as “Laplacin-of-Guassin” and “palys,” along with other inconsistencies. We appreciate your attention to detail and have ensured that the revised manuscript is clearer and more precise.

Blue: Responses

Cyan: Revised texts from last time

Red: Revised texts for this time

Point-to-point Responses to the Reviewer #3:

I am impressed with the effort the authors have put into revision. The paper is now much improved.

When it comes to ribosomal stalling on charged motifs - and cellular factors that can assist the ribosome in negotiating these - there are a couple of recent papers are relevant and worth citing:

Resolution of ribosomal stalling by EF-P and ABCF ATPases YfmR and YkpA/YbiT. Hiraku Takada, Keigo Fujiwara, Gemma C Atkinson, Shinobu Chiba, Vasili Hauryliuk. Nucleic Acids Research, Volume 52, Issue 16, 9 September 2024, Pages 9854–9866

The ABCF proteins in Escherichia coli individually cope with 'hard-to-translate' nascent peptide sequences. Yuhei Chadani, Shun Yamanouchi, Eri Uemura, Kohei Yamasaki, Tatsuya Niwa, Toma Ikeda, Miku Kurihara, Wataru Iwasaki, Hideki Taguchi. Nucleic Acids Research, Volume 52, Issue 10, 10 June 2024, Pages 5825–5840

Response: Thank you for your positive feedback and for recognizing the improvements in the revised manuscript.

We appreciate your suggestion regarding the relevance of recent studies on ribosomal stalling on charged motifs and the role of cellular factors. We have reviewed the recommended papers and agree that they are highly relevant to our work. Accordingly, we have cited these studies in the revised manuscript and discussed their contributions in the context of our findings.

The revised introduction:

Here, we reported that OLA1/YchF binds to the ribosomal large subunit and promotes the efficient translation on D/E-rich mRNA. Disruption in the translation can be caused by the

truncated mRNA or mRNA with a specific structure/sequence[29]. Rescuing the stalled ribosome on these mRNAs need numerous ribosome-associated proteins to release the nascent peptide and split the ribosome. For instance, the proteins Dom34, Hbs1 and Rli1 target ribosomes with truncated mRNA in eukaryotes, catalyzing the splitting of the subunit[29]. While stalls on specific mRNA structure/sequence can be resolved under optimal conditions, i.e., synthesis of P-rich is made possible by EFP (in bacteria, eIF5A in eukaryotes and archaea)[30]. Stalling on R/K-rich mRNA is promoted by Rbg1/Tma46 complex or target/split by RQT complex if ribosome collision occurs[31,32]. The bacteria ATP-Binding Cassette family-F (ABCF) proteins, YheS, YbiT/YkpA, EttA and Uup/YfmR could cope with these problematic nascent peptide sequences within the exit tunnel[33,34]. The ATP hydrolysis-coupled structural rearrangement is critical for handling ribosome stalling caused by nascent peptides[33]. Currently, there is limited research on D/E-rich mRNA. Through structural analysis and Ribo-seq data, we speculated that OLA1/YchF can enhance translation on D/E-rich mRNA, a notion validated by our constructed reporter. OLA1/YchF binds to the large subunit at the uL14/bL19 position via its helical domain, preventing the re-association of split ribosomes. These findings not only provide deeper insights into OLA1/YchF function but also contribute in better understanding the regulation of translation on D/E-rich mRNA.

The revised discussion:

It has been reported that 63% of the conserved stall sites (CSS) could be explained by specific amino acids (Pro, Gly and Asp) at P-site, Glu at A-site, lysine stretches or negatively charged amino acids at the entrance to the exit tunnel[48]. As the entrance to the exit tunnel is narrow, it has been hypothesized that upon encountering it, the negative charge of amino acids might repel the negative charge of the exit tunnel and slow down the translation[48,60]. EF-P/eIF5A, Rbg1/Tma46, and some ABCF proteins have been reported to promote the translation on these stall sites[31,33,34].

Point-to-point Responses to the Reviewer #1:

Reviewer #1 (Remarks to the Author):

After confirming the splitting activity of YchF, the only comment is that the authors should be clear about the fact that the 50S complex likely resembles the post splitting complex and that this might be different than the 70S encounter complex.

Response: Thank you for your suggestion. We have added some description to the final manuscript.

"This suggests that the YchF/50S structure we obtained likely resembles the post-splitting complex, which may differ from the 70S encounter complex."

Point-to-point Responses to the Reviewer #2:

The authors addressed most of my comments and improved the manuscript. It is great to see that additional experiments have been performed and contributed to this work. Two inconsistencies, however, still need to be addressed to ensure the study is rigorous.

1. The BSA of 643 Aˆ2 usually (but not always, as authors correctly note in their response) indicates weak to modest binding between macromolecules. The authors modified their conclusion to "This accounts for a reasonable interface among protein complexes30,31, suggesting a stable binding of YchF to the 50S subunit."

Based on their own measurements of YchF action on 70S dissociation, however, they concluded that "Lower amount (5-fold excess) of YchF did not increase the splitting of 70S. These results indicated that YchF binds weakly to the purified ribosomes or subunits, which is consistent with the binding assay shown in Extended Data Fig. 3a."

This inconsistency is easy to resolve by interpreting the BSA calculations as "This accounts for a lower to modest interface among protein complexes 30,31, suggesting a weak or modest binding consistent with our experimental data discussed below".

Response: Thank you for your suggestion. The revised text is: "This accounts for a lower to modest interface among protein complexes[38,39], suggesting a weak or modest binding consistent with our experimental data discussed below."

2. The response to my point about Fig. 6c is inadequate: the data for human cells do not support the conclusion that OLA1 affects the D12 reporter because the difference between WT and OLA-/- is very small and the spread overlaps. The authors should clearly point out that the effect of OLA1 in human cells

was not as conclusive as for Ychf in E. coli cells, possibly due to the complex regulatory mechanisms in human cells.

Response: Thank you for your valuable suggestion. The revised text is:

"Results showed that deletion of *ychF* caused a significant increase in GFP/mCherry level, which can be interpreted as activation of the ribosome-associated quality control (RQC) pathway, leading to degradation of the mCherry protein. In $OLA1^{-/-}$ cells, an upward trend was also observed. However, the difference between WT and $OLA1^{-/-}$ cells was very small, and the spread overlapped, indicating the effect of OLA1 in human cells was not as conclusive as that of YchF in *E. coli* cells, possibly due to the more complex regulatory mechanisms in human cells."

3. Proofreading of the manuscript is still necessary, as numerous typos and stylistic errors must be fixed. Another suggestion is to use the present tense for new findings instead of the past tense (e.g. "Here, we reported …").

Response: Thank you for the feedback regarding proofreading and tense usage in the manuscript. We have carefully reviewed the text again to address all typos and stylistic issues. Additionally, we have adjusted the use of tense, ensuring new findings are consistently presented in the present tense as recommended.