The yeast *FIT2* homologs are necessary to maintain cellular proteostasis and membrane lipid homeostasis

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MS TITLE: Yeast FIT2 homolog is necessary to maintain cellular proteostasis and membrane lipid homeostasis

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

here are some suggestions for further experiments and, while I agree that these would enhance the work overall it may be that you are currently unable to conduct them in a suitable time frame. Overall I consider that you will likely be able to address each of the comments with some clarification and amendment to the text. The major issues raised relate more to the overall structure of the paper and flow of the text.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Shyu and colleagues investigates the role of scFIT proteins in lipid droplet morphology, the unfolded protein response, and ER-associated protein degradation. This study made several interesting observations that were done with high scientific rigor, however the data were somewhat disjointed from figure to figure, and it was unclear what the specific “unknowns” were to be addressed. From that standpoint, it was difficult to assess how the data advanced the specific unknowns. From the introduction (lines 76-80), it seemed the major questions could be stated thusly: Do scFIT proteins bind and partition NLs or influence NL and phospholipid metabolism?
How are these functions relevant to cellular function and broader physiological processes? Based on these this, it was surprising that the role of scFIT proteins in lipid metabolism was not pursued. Instead, the effects of the scFITs on protein degradation, while interesting, seemed more modest and somewhat variable (compare Fig. 4B vs 5A). The authors are to be commended for an overall interesting and rigorous study, but perhaps some additional considerations could aid in the cohesion of the manuscript.

Comments for the author

Major comments/questions:

In figure 2A, modulating IRE1 levels in scs3-depleted cells makes it difficult to determine the role of scs3 in UPR-induced TG accumulation. Would it not be more informative to determine the levels of TG trapped in the ER by comparing IRE1Δ vs IRE1Δ-scs3-1? The nice EM’s in Fig. 2B suggest that while UPR disruption induces LD accumulation, the SCS3 is required for triglyceride exit from the ER and into cytosolic LDs. Also based on these EM’s it is very curious why an increase in ER TG levels is not observed in SCS3-depleted cells compared to WT.

While modulating SCS3 clearly exhibited dramatic effects on LDs and PC levels (Fig. 2), the authors downplayed the discovery of four interacting proteins identified in lipid metabolism (Fig. 3). Instead, four other proteins involved in ubiquitin/proteasomal degradation were highlighted. It was unclear why this decision was made, especially since the lipid metabolism enzymes bound to SCS3 at nearly equivalent levels as those involved in protein degradation.

What is the proposed mechanism for how scFIT proteins regulate ER lipids, and how might the physical interaction between scFITs and ERAD proteins influence this?

Reviewer 2

Advance summary and potential significance to field

FIT2 proteins have been implicated in lipid droplet (LD) extrusion from the ER, lipid phosphohydrolase activity, and phospholipid metabolic control, but exactly how these proteins perform all these functions is far from clear. The manuscript submitted by Shyu et al. attempts to cast light on these mysterious proteins concentrating mainly on one of the two yeast FIT2 proteins, Scs3. The authors start by exploring the known synthetic lethality between Scs3 and the ER unfolded protein response (UPR) sensor, Ire1. They show a strong interaction between Scs3 and the UPR. SCS3 is markedly upregulated by tunicamycin (which interferes with ER protein glycosylation

and protein folding) and scs3Δ cells are very sensitive to tunicamycin providing good evidence that Scs3 acts to dampen ER stress. To understand the relationship of IRE1 and SCS3, the authors made a temperature-sensitive scs3 mutant (scs3-1). At the restrictive temperature for scs3-1 in a strain lacking IRE1, lipid droplets are smaller and flattened in the ER. More neutral lipid copurifies with microsomes, suggesting altered ER lipid composition. Changes in phospholipid composition as a function of inositol and choline supplementation is very different in scs3Δ compared to wt. (Low inositol normally stimulates phospholipid synthesis, and choline provides substrate for PC synthesis via the Kennedy pathway). For example, PC synthesis is much higher after inositol supplementation in the scs3Δ strain compared to wt, and removal of inositol evokes a difference response in PC synthesis in the mutant strain. In order to probe mechanism, the authors next did an unbiased membrane two-hybrid experiment. While this was not helpful regarding phospholipid regulation, they found several hits in the proteostasis ysstem.
Following up on this, they found some instances where model substrates for the proteosome were more stable when both yeast FIT2 proteins were deleted. (The effects were more modest to none when only SCS2 was deleted.). The rate of proteolysis was also affected by exogenous choline levels, linking changes in lipid metabolism and proteostasis to the FIT2 proteins.

There is considerable interest in the mechanism of action of FIT2. Although the purified protein binds to lipids such as di- and triacylglycerols, and a phosphohydrolase catalytic site seems intact in the protein (the lipid substrate may not yet have been discovered), exactly how this protein provides its functions is far from clear. This manuscript fleshes out the known genetic interaction between IRE1 and SCS3 (i.e., synthetic lethality), showing that Scs3 when present increases the rate of protein degradation in some cases and increases multiubiquitination. Although there really is no molecular mechanistic information here, it also links a role for Scs3 in proteostasis with phospholipid metabolism, as well as with lipid droplet morphology and ER LD trapping. The big mystery of how FIT2 fulfills these functions remains, but there now are more pieces to the puzzle. The findings will be of significant interest to the expanding community interested in lipid droplets and metabolism.

Comments for the author

(1) In Fig 1B, increase in SCS3 mRNA levels by Tm is compared with an LBS condition, and not with a more unstressed condition. It seems that this is an important control.
(2) You may want to point out that even at 25 °C, the scs3-1 cells seem to grow really poorly (Fig 1E).
(3) Line 134: The statement that “IRE1 is not essential in the absence of YFT2” should be referenced.
(4) Line 144: Perhaps substitute “tightly integrated in the ER membrane” for “tightly associated with the ER”.
(5) Fig 2A: Could the increase in microsomal TG be caused simply by the smaller integrated LDs not pulling off from the membrane during centrifugation, rather than more TG dispersed in the ER? Also, as the scs3-1 cells seem on the road to death under the restricted conditions, can this result be attributed to dying cells? A control for this would help.
(6) It was really hard for me to relate the data in Fig 2E to data in Fig. 2C and 2D. Or maybe they are not relatable as choline remains constant in 2E.
Do the authors have any thoughts about how Pup1, a proteosomal 20S core protein, can physically interact with Scs3? Seems a stretch.
(7) Fig. S7: Pgc1 seems to have an INCREASED degradation in ScFIT2Δ. How is this explained?

First revision

Author response to reviewers' comments

Please note that most changes are highlighted in red throughout the revised manuscript.
We would like to thank the reviewers and the editor for taking the time to evaluate our original manuscript submission. Your comments and feedback were seriously considered, and we believe

that our manuscript is consequently improved as a result. Please find the detailed responses to your comments below.

Reviewer #1 comments

1. From that standpoint, it was difficult to assess how the data advanced the specific unknowns. From the introduction (lines 76-80), it seemed the major questions could be stated thusly: Do scFIT proteins bind and partition NLs or influence NL and phospholipid metabolism? How are these functions relevant to cellular function and broader physiological processes? Based on these this, it was surprising that the role of scFIT proteins in lipid metabolism was not pursued. Instead, the effects of the scFITs on protein degradation, while interesting, seemed more modest and somewhat variable (compare Fig. 4B vs 5A).

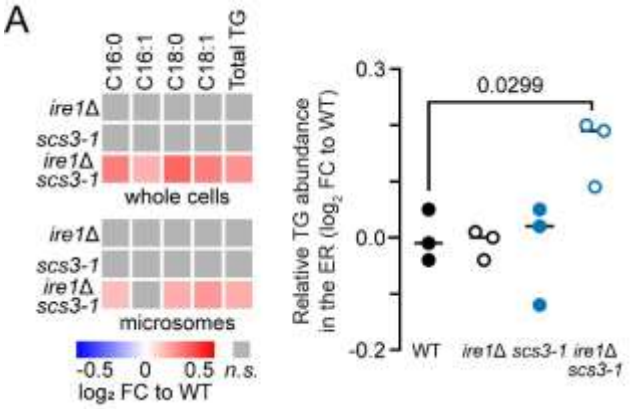
We thank the reviewer for highlighting the issue in the introduction. We have modified the last sentence of this paragraph to "Moreover, how any of these functions impact ER homeostasis and the UPR are partially unexplored" (lines 80-81).

We also agree with the reviewer that the variation in protein degradation might seem modest between Fig. 4B and 5A. However, we observed a consistent decrease in the degradation of model substrate in *ScFITΔ* strain compared to WT either in SC media or in SC media lacking inositol and supplementing with choline. On the other hand, the overexpression of *SCS3* was sufficient to rescue the defect in protein degradation especially for the soluble model substrate CPY*. It should be noted that we used cycloheximide assay to monitor the degradation of the model substrates. The half-life of proteins cannot be directly measured as it includes steady state abundance of the proteins while pulse-chase assay provides a direct measurement of protein's half-life (PMID 27167179). Therefore, someone might expect to detect larger differences in protein degradation using pulse- chase in the *ScFITΔ* strain.

2. In figure 2A, modulating IRE1 levels in scs3-depleted cells makes it difficult to determine the role of scs3 in UPR-induced TG accumulation. Would it not be more informative to determine the levels of TG trapped in the ER by comparing IRE1Δ vs IRE1Δ-scs3-1?

As suggested by the review, we have repeated the assay by including the control strain *ire1Δ*. We have updated Fig. 2A and we have included the image of the thin layer chromatography (TLC) plate in Fig. S4.

New Figure 2A:



3. The nice EM's in Fig. 2B suggest that while UPR disruption induces LD accumulation, the SCS3 is required for triglyceride exit from the ER and into cytosolic LDs. Also based on these EM's it is very curious why an increase in ER TG levels is not observed in SCS3-depleted cells compared to WT.

We have repeated the quantification of TG levels by including the missing control strain *ire1Δ*. We observed a significant increase in TG levels only in the strain *ire1Δscs3-1* but not in *scs3-1* when compared to WT. We agree with the reviewer that someone should expect to see a

significant increase of TG levels in *scs3-1* as suggested by the TEM images. However, the other TEM images of *scs3-1* and *ire1Δscs3-1* in Fig. 5B suggest that overall *ire1Δscs3-1* cells contain more LDs associated to the ER. Therefore, these observations are in agreement with Fig. 2A.

4. While modulating SCS3 clearly exhibited dramatic effects on LDs and PC levels (Fig. 2), the authors downplayed the discovery of four interacting proteins identified in lipid metabolism (Fig. 3). Instead, four other proteins involved in ubiquitin/proteasomal degradation were highlighted. It was unclear why this decision was made, especially since the lipid metabolism enzymes bound to SCS3 at nearly equivalent levels as those involved in protein degradation.

We agree with the reviewer that we failed to provide a logical transition between Fig. 1-2 and Fig. 4-5. From our MYTH data (Fig. 3), we explored 2 different avenues including the one presented in Fig. 4 and 5. The other avenue we took was based on previous findings linking LD to Doa10-depedent ERAD substrate (PMID 27357570). The authors reported that Doa10 regulates the levels of some LD proteins through the ERAD pathway including Pgk1 and Yeh1. Therefore, we asked if ScFIT proteins play a role in regulating these native substrates. We also included Doa10-depedent native substrate Sbh2 that we have used for another publication (PMID 31201345). The degradation rates of Sbh2 and Yeh1 were similar in WT and in *ScFITΔ* strain while the steady state of Yeh1 was noticeably lower in *ScFITΔ* strain (Fig. S7A,B). On the other hand, the degradation rate of Pgc1 was significantly accelerated in *ScFITΔ* strain (Fig. S7C). Along with the identification of Pgc1 as a Doa10-dependent ERAD substrate, its proper localization dynamics between the ER and LD membranes was found to be critical in determining its stability (PMID 27357570). Doa10 reportedly recognizes ER-localized Pgc1 through its hairpin loop, which then serves as a degron that concentrates Pgc1 on the surface of LDs. As LDs fail to properly mature and remained tethered to ER membrane in the absence of the ScFIT proteins, the lateral diffusion of the pool of Pgc1 proteins to the ER may be increased in *ScFITΔ* mutants, resulting in continual degradation by Doa10. We hypothesized that native proteins in their proper conformation may not illicit a proteotoxic effect on *ScFITΔ* cells, and that an otherwise compromised protein degradation pathway in this mutant could remain fully capable of clearing these endogenous proteins. From here, we reasoned that ScFIT proteins promote the degradation of misfolded proteins. This line of thought let us the findings presented in Fig. 4 and 5. As suggested by the reviewer, we should have explored the link between lipid metabolism related proteins identified from our MYTH to ScFIT proteins. This will be explored in future studies.

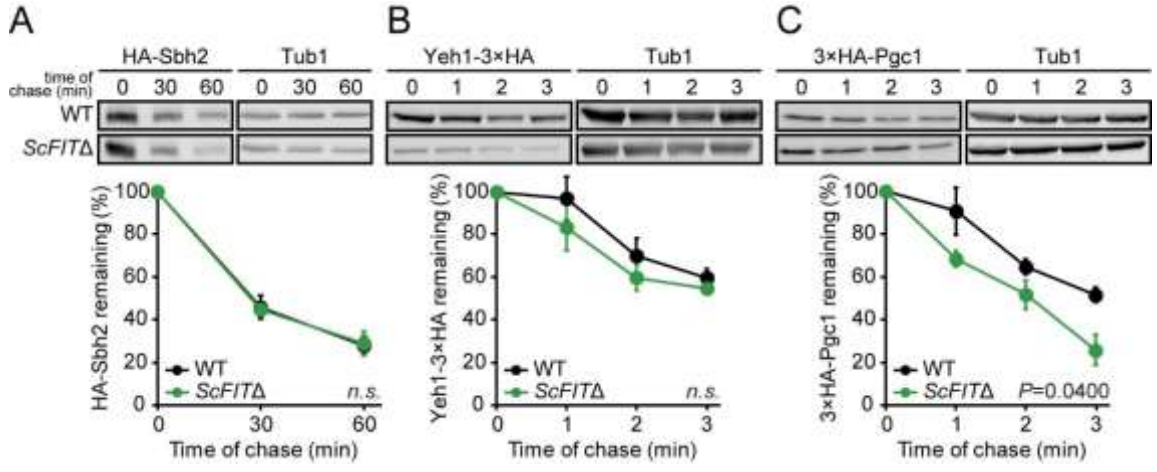


Figure S7, Refers to **Figure 4. ScFITΔ mutants can degrade natively folded proteins.** (A-C) Protein levels of the known Doa10-dependent ERAD substrates HA-Sbh2 (A), Yeh1-3×HA (B), and 3×HA-Pgc1 (C), were monitored at the indicated time points following the attenuation of protein translation with cycloheximide. Chase experiments for B and C were done with cells grown in media supplemented with 0.1% oleic acid. Data shown are the mean ± SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

5. What is the proposed mechanism for how scFIT proteins regulate ER lipids, and how might the physical interaction between scFITs and ERAD proteins influence this?

We invite the reviewer to our response of Reviewer #2, comment #7.

Reviewer #2 comments

1. In Fig 1B, increase in SCS3 mRNA levels by Tm is compared with an LBS condition, and not with a more unstressed condition. It seems that this is an important control.

We are sorry to see that our graph or figure legend might have been unclear. SCS3 and YFT2 mRNA levels treated with tunicamycin (Tm) or in the absence of inositol (-ino) have been normalized to untreated WT cells. Compared to untreated WT cells, *SCS3* mRNA levels are 6.4 and 1.5 times higher in Tm and -ino conditions, respectively, which we transformed to log2 of these fold changes (FC) to obtain 2.6 and 0.629, respectively (WT untreated becomes zero).

2. You may want to point out that even at 25 °C, the scs3-1 cells seem to grow really poorly (Fig 1E).

We have repeated the growth assay of *scs-1* and *ire1Δscs3-1*. As noted by the reviewer, *ire1Δscs3-1* growth at 25ºC is still poor. However, we were confident that *scs3-1* is still functional, at least in part, at 25ºC as we could observed a different phenotype at 37°C as well as depending on *IRE1* to maintain its growth at 37ºC but not at 25ºC (data not shown).

3. Line 134: The statement that "IRE1 is not essential in the absence of YFT2" should be referenced.

We thank the reviewer for point this out. We were not referring to a publication but rather to our assay demonstrating that *ire1Δyft2Δ* cells were easily dropping the plasmid containing a functional *IRE1* gene (Fig. S3B), demonstrating the absence of synthetic lethality between the two genes. We have rephrased the sentence for clarity.

Updated manuscript (lines 135-136):

"As IRE1 is not essential in the absence of YFT2 (Fig. S3B), a temperature sensitive allele of YFT2 could not be generated."

4. Line 144: Perhaps substitute "tightly integrated in the ER membrane" for "tightly associated with the ER".

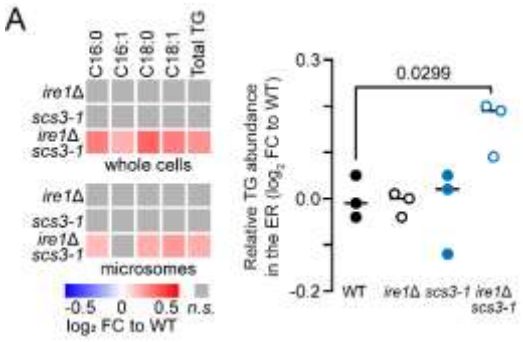
We have modified the manuscript accordingly.

5. Fig 2A: Could the increase in microsomal TG be caused simply by the smaller integrated LDs not pulling off from the membrane during centrifugation, rather than more TG dispersed in the ER? Also, as the scs3-1 cells seem on the road to death under the restricted conditions, can this result be attributed to dying cells? A control for this would help.

The reviewer point is valid that the accumulation of TG at the ER could be due to LDs that haven't budded off the membrane. However, we cannot exclude that some of accumulated TG might be dispersed at the ER membrane. Someone can speculate that high levels of TG at the ER membrane might cluster between the two-lipid leaflet to form a lens like shape as proposed in the early stage of lipid droplet formation. It is also clear from our findings that the UPR regulates TG levels at the ER.

As suggested by the review, we have repeated the assay by including the control strain *ire1Δ*. We have updated Fig. 2A and we have included the image of the thin layer chromatography (TLC) plate in Fig. S4.

New Figure 2A:



6. It was really hard for me to relate the data in Fig 2E to data in Fig. 2C and 2D. Or maybe they are not relatable as choline remains constant in 2E.

We agree with the reviewer that it might be confusing as we failed to specify in Fig. 2E if choline was present for the experiment. Fig. 2E should be compared to Fig. 2D as t=0 cells are grown in the absence of inositol (-ino) and with choline until t=180 where inositol is added to the media (+ino) while choline remains present as well. We have modified Fig. 2 legend accordingly.

7. Do the authors have any thoughts about how Pup1, a proteosomal 20S core protein, can physically interact with Scs3? Seems a stretch.

Although we can't totally exclude unspecific interactions, the MYTH screen was carefully optimized to minimize unspecific interactions with different optimization steps including the use of 3-amino-1,2,4-triazole (3-AT). The validation by retransforming the isolated plasmid (in that case Pup1) in the bait strain was carried out in the presence of X-gal to provide a second layer of specificity in the interaction between the bait and the pray (Fig. S5A). This was done in comparison to a yeast strain expressing an unrelated negative control bait construct encoding for the human CD4 T-cell surface glycoprotein. We apologize that the MYTH screen method was missing in the submitted manuscript. It has been added to the current version. Based on these approaches, we are confident that the *in vivo* interaction we detected is specific, but we cannot exclude that Pup1 was picked up to its possible proximity to Scs3 without physical protein-protein interactions. We can speculate that the N-terminus of ubiquitin (NubG-Pup1) was exposed on the surface of the proteasome and that a proteasome subpopulation was in proximity of the ER perhaps through the ERAD pathway. Further investigation will be needed in the future to better understand the relationship (or the absence of) between Pup1 and Scs3. We removed the sentence referring to Pup1 from the manuscript.

Updated manuscript (lines 215-218):

"Doa10 is one of the key E3 ubiquitin ligases in yeast, and is involved in ER-associated degradation (ERAD) of proteins. Taken together, these suggest that Scs3 may function to a certain extent in protein quality control pathways, specifically in the UPS"

8. Fig. S7: Pgc1 seems to have an INCREASED degradation in ScFIT2Δ. How is this explained?

We were also surprised by this finding. So far Pgc1 is the only ERAD model substrate where we observed a degradation rate increase in *ScFIT*Δ. Although the degradation trend is the opposite of the other ERAD substrates, we thought it was best to include it in our manuscript instead of omitting it as it might be of interest to other research groups. Not all ERAD substrates are equal especially when comparing native protein turnover to unfolded protein model substrates. The degradation of unfolded proteins in the ER mostly depend on the fitness of the ERAD machinery while some unfolded proteins might be exclusively degraded through the vacuole or only when ERAD is defective. On the other hand, native proteins like Pgc1 are mostly prone to degradation when they (1) are damaged, (2) fail to be part of their respective protein complexes, (3) regulatory proteins levels of the native proteins change, or (4) protein folding and quality control is compromised.

In the result section of the manuscript, we speculated on why Pgc1 might be degraded faster in *ScFIT*Δ. It would be interesting to investigate a wide range of native model substrates degradation rate in *ScFIT*Δ as well as dissecting the mechanism leading to the fast degradation of Pgc1 in the absence of *SCS3* and *YFT2* in future studies.

Second decision letter

MS ID#: JOCES/2020/248526

MS TITLE: Yeast FIT2 homolog is necessary to maintain cellular proteostasis and membrane lipid homeostasis

AUTHORS: Wei Sheng Yap, Peter Jr. Shyu, Maria Laura Gasper, Stephen A Jesch, Charlie Marvalim, Will Prinz, Susan A Henry, and Guillaume Thibault
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This paper describes a novel role for a family of fat storage inducing transmembrane (FIT) proteins in S. cerevisiae. Traditionally known for their role in lipid droplet biogenesis, FIT proteins SCS3 and YFT2 were shown to mediate lipid metabolism, phospholipid homeostasis, and proteostasis, particularly during ER stress. In the absence of FIT proteins, lipid accumulates within the ER due to impaired LD biogenesis, resulting in ER stress-induced triglyceride accumulation and a reduced capacity to degrade misfolded proteins. This study highlights the interconnected nature of lipid droplet biogenesis, metabolism, and protein quality control.

Comments for the author

The authors have met all of my original concerns. Congratulations on a beautiful work.

Reviewer 2

Advance summary and potential significance to field

I think these observations are sound and that a link between the FIT2 protein Scs3 and ERAD is interesting and adds to our knowledge of FIT2 function. I am satisfied with the authors' response to my comments, and it seems that their response to the other reviewer are reasonable as well. I have no further critical comments regarding this revision.

Comments for the author

No other suggestions.